REGULATION OF MAEDI-VISNA VIRUS AND CYTOKINE GENE EXPRESSION IN MACROPHAGES

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Declaration

The results in thesis and its composition are solely the work of the author, except where otherwise indicate and all work of other authors is acknowledged.

Abstracts

Macrophages are proposed to play a central role in Maedi-visna virus (MVV) infection as target cells. However, the level of MVV load in alveolar macrophages (AMs) and its correlation with pulmonary lesions is unknown. Also it is not yet clear whether cytokine expression is dysregulated in macrophages infected with MVV. In addition, the effects of exogenous cytokines on MVV replication in macrophages have not been documented. The aims of this study were to determine 1. the level of MVV DNA in AMs compared with blood monocytes in natural infection and assess the correlation of viral burden and replicative status to the lung lesions related to MVV infection; 2. the differential expression of cytokine genes in macrophages infected with MVV *in vivo* and *in vitro*; 3. the effect of GM-CSF, TGF-β and IFN-γ on MVV replication in macrophage *in vitro*

Quantitative competitive-PCR for MVV *pol* was developed to quantify accurately the level of MVV DNA load in alveolar macrophages (AMs). This first required constructing a competitive template which bears the same recognition site as target template to be quantified, but truncated by a 25 bp sequence. The quantitative evaluation of the PCR signal clearly shows that the overall levels of MVV DNA load in AMs was significant higher than that in blood monocytes ($P<0.05$). Changes of viral load in AMs differed in relation to the histopathological lesions in the lungs. Relatively high viral load was found in AMs from the lung with histopathological lesions. Furthermore, MVV replication did not occur in AMs isolated from sheep without histopathological lesions in the lung, but could stimulated *in vitro*, suggesting that the levels of MVV DNA load reflects the pathological manifestation in the site of disease. In addition, MVV replication in AMs *in vivo* may require the availability of certain factors to activate viral replication and these may be important in disease progression. Such factors could be cytokines produced by macrophages present or recruited in the site of disease.

Determination of cytokine expression in response to MVV infection *in vivo* and *in vitro* show a significant increase in the expression of IL-6, IL-10, GM-CSF and TGF-β mRNA in AMs isolated from sheep naturally infected with MVV. The level of GM-CSF mRNA was found to be much higher in AMs isolated from sheep with lung lesion when compared with those without lung lesions ($P<0.05$), but the levels of IL-10 and IL-6 mRNA expression in AMs had no association with histopathological findings in the lung. TNF-α and TGF-β mRNA level were not altered significantly in AMs. Blood monocytes showed a similar pattern of cytokine transcripts to AMs. In lymph nodes, the levels of IL-10, IL-2, and IL-6 mRNA were found to be higher than those in seronegative controls, whereas the levels of GM-SCF mRNA werenot significantly altered.IFN-γ mRNA was undetectable in both groups. Interestingly, IL-10 mRNA expression in monocyte-derived macrophages (MDMs) was altered in response to MVV infection *in vitro*. This result raised a possibility that direct infection of MDMs with MVV may be not sufficient to stimulate detectable levels of IL-10 mRNA in these cells. In analysing heterogeneity of cytokine function in MVV infection increased expression of IL-6 was detected in MDMs exposed to GM-CSF but not to MVV, suggesting that interaction of MVV with the target cells is not

essential for the expression of IL-6. In determining the effects of GM-CSF, TGF-β and IFN-γ on the replication of MVV in MDMs, the enhancing effect of GM-CSF and inhibitory effects of TGF-β on MVV replication in MDMs was observed whereas IFN-γ was unable to demonstrate any apparent enhanced or inhibitory effects of IFN-γ on MVV replication in MDMs. Cytokines modulation of surface antigens expression on macrophages was also investigated IFN-γ had an enhanced effect on the expression of MHC class I and II on MDMs whereas GM-CSF was showed to have an enhanced effects on MHC class I but not on MHC class II. Both cytokines did no significantly increased the expression of CD14, CD45 and CD11c, and had no influence on the expression of CD1b and CD11b on MDMs and MVV-infected MDMs.

These results may have important implications in understanding of regulation of MVV in macrophages as target cell for MVV and the balance between stimulatory and inhibitory cytokines that may play a critical role in controlling MVV replication and expression and subsequently in the clinical progression of MVV infection.

Dedication

I would like to dedicate this thesis to my parents, Fufen Yang and Yutai Zhang; my wife, Yanmin Li; my son, Yuen Zhang.

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AGID:	agar gel immunodiffusion test
AIDS	aquired immunodeficiency syndrome
AM	alveolar macrophage
AP	alkaline phosphatase
ATPase	adenosinetriphosphatase
BALF	bronchoalveolar lavage fluid
BSA	bovine serum albumin
CAEV	caprine arthritis-encephalitis virus
CD	cluster of differentiation
cDNA	DNA complimentary to mRNA
CHAPs	3-[(-cholamidopropyl)dimethylammonio] -1-propane sulphonate
CNS	central nervous system
ConA	concanavalin A
CTL	cytotoxic lymphocyte
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DNTP	deoxynucleoside -triphosphate
DOC	deoxycholic acid
DMEM	Dulbecco's modification of Eagle's medium
DMF	dimethylforinamide
DMSO	dimethyl sulphoxide
ds DNA	double stranded DNA
EDTA	ethylenediaminetetra-acetate
ELISA	enzyme-linked immunosorbant assay
env	envelope protein
FCS	foetal calf serum
FACS	fluorescent activated cell sorter
FITC	flourescein isothiocynate
FIV	feline immunodeficiency virus
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBSS	Hank's buffered salt solution
HIV	human immunodeficiency virus
IFN	interferon
IL	interleukin
LIP	lymphocytic interstitial pneumonia
LN	lymph node
LPS	lipopolysaccharide
McAb	monoclonal antibody
MDM	monocyte-derived macrophage
Meth	methicillin
MHC	major histocompatabilty complex
MVV	maedi-visna virus
orf	open reading frame
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline

PCR	polymerase chain reaction
QC-PCR	quantitative competitive PCR
RNase	ribonuclease
s.d.	standard deviation
SDS	sodium dodecal sulphate
SDW	sterile distilled water
SIV	simian immunodeficiency virus
ss RNA	single stranded R.NA
$TCID_{50}$	50% tissue culture infectious doses
TAE	Tris acetate EDTA
TEMED	N,N,N',N'-tetramethylethylenediarnine
TGF-β	transforming growth factor beta]
RT-PCR	reverse transcription PCR
U.V.	ultra-violet

CHAPTER 1

LITERATURE REVIEW

1. 1: **Maedi-Visna virus**

1.1.1: **Introduction**

Maedi-Visna virus (MVV) is the prototype virus of the retroviral subfamily Lentivirnae, the members of which include the human, simian, bovine and feline immunodeficiency virus, caprine arthritis encephalitis virus (CAEV), and equine infectious anaemia virus (EIVA) and cause a chronic multiorgan disease characterised by long incubation periods and slowly progressive clinical course. MVV is widespread in sheep populations. Like other lentiviruses, MVV infects monocytes/macrophages and causes persistent infection. However, unlike the human and simian immunodeficiency viruses (HIV and SIV), MVV does not cause immune deficiency but instead causes chronic mononuclear inflammation of various tissues (Gendelman *et al.* 1989, Brodie *et al.* 1995). The progressive interstitial pneumonia is the most obvious manifestation of MVV-induced inflammation. The infected sheep also frequently have indurative mastitis and non-suppurative arthritis and more rarely wasting and paralysis caused by viral meningo-encephalitis (Sigurdsson 1954, Gudadottir 1974, Cutlip *et al.* 1977, De Boer *et al.* 1979, Haase 1986, Narayan *et al.* 1988, 1989, Houwers 1989, Simard *et al.* 1990a, 1990b, Brodie *et al.* 1992, 1993, 1994, 1995, Kwang *et al.* 1993). The lesions caused by MVV are characterised by chronic active inflammatory changes with mononuclear cell infiltration and proliferation (Lairmore *et al.* 1986, 1988a, Watt *et al.* 1994). However, the precise mechanisms by which MVV causes damage remain to be determined.

1.1.2: **Structure of MVV**

MVV is an enveloped virus, with an RNA genome, and exhibits typical retrovirus morphology (Harter 1976). The mature virion has a diameter of approximately 100 nm with a cone-shaped core composed of the viral gag protein p25. Inside this nucleoid contains a positive sense single-stranded RNA (ssRNA) (Brahic *et al.* 1977) with which the viral RNA-dependent DNA polymerase (POL, also called a reverse transcriptase) and nucleoprotein (p14) are closely associated (Lin & Thormar

1970). The inner portion of viral membrane is surrounded by gag protein p16 that provides the matrix (MA) for the viral structure and is vital for the integrity of the virions. It has been demonstrated in HIV that MA is required for incorporation of the Env proteins into mature virions. The viral surface is surrounded by a host-cell-derived envelope from which project viral glycoprotein spikes composed of gp135 and gp41 (transmembrane protein, TM). Parts of the central and NH2-terminal portions of gp41 are expressed on the outside of the virions and the central region of gp41 binds to the external viral gp135 in a noncovalent manner. The virion gp135 located on the viral surface contains the binding site for the cellular receptor (s) and the major neutralizing domains.

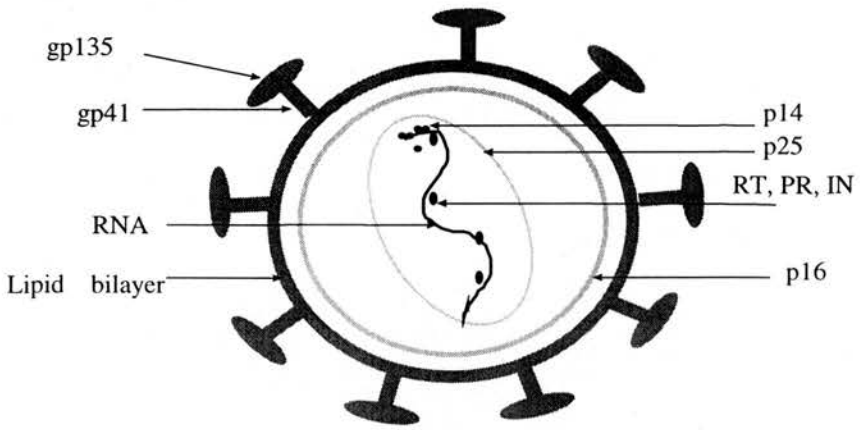


Figure 1.1 A MVV virion with the structural and other proteins identified.

gp135: envelope surface protein, gp41: envelope transmembrane glycoprotein, p16: matrix protein, p25:capsid structural protein

1.1.3: **Molecular biology of MVV**

The genomes of MVV, like other lentiviruses, are positive-stranded RNA molecules that is approximately 9.2 kilobases and exists within the virion as a dimer with two identical RNA molecules (Sonigo *et al.* 1985, Brawn *et al.* 1987, Querat *et al.* 1990, Sargan *et al.* 1991, Staskus *et al.* 1991). The genome of the virus contains the structural genes (*gag* and *env*) and enzymatic gene (*pol*) common to all retroviruses.

However, in addition to these genes, MVV genomes encode regulatory genes which are not found in other non-lentiviruese retroviruses (Carey and Dalziel 1993).

The RNA genomes of MVV contain repeated regions (R) at the 5' and 3' ends and unique 5' and 3' (U5 and U3) that are important in the process of reverse transcription of the genomic RNA into DNA. These regions form the long terminal repeats (LTR) found in all other retroviruses. The U3 region of LTR contains the transcription control elements (enhancer and promotor elements) that control the transcriptional activity (Carruth *et al.* 1994). The enhancer elements are recognised by cellular transcription factors that activate basal transcription of viral RNA. The viral RNA transcripts initiate transcription at the cap site (+1) in the 5' LTR and terminate in the 3' LTR at the polyadenylation signal. A particular lentivirus uses a specific cellular transcription factor due to its cellular tropism. Thus, SIV uses NF-(B, a transcription factor present in activated T cell and macrophages (Carruth *et al.* 1994, Toohey *et al.* 1994). In contrast, MVV uses the transcription factor c-Jun and c-Fos present in macrophages. These factors were found to bind to an AP-1 site in the MVV LTR (Shih *et al.* 1992). Recently, a transcription factor related to the AML/PEBP2/CBF superfamily was found to bind to AML (vis) site in LTR of MVV EV1 which lack AP-1 site in LTR (Sutton *et al.* 1997). In addition to the identified transcription factors, it assumed that there are sites for additional cellular factors in the U3 region of all lentiviruses. These factors may play an important role in the virus life cycle by providing the virus with the capacity to be activated at the transcriptional level in different cellular environments.

1.1.3.1: **Structural genes of MVV**

The viral gag, env and pol, which lie in the usual order flanked by LTR, encode proteins required for production of infectious virus particles. The gag (group-specific antigen) gene encodes a 55 kD non-glycosylated precursor core polypeptide (p55) which is cleaved after translation to form nucleocapsid p25, p16 and p14 (nucleoprotein). The principal gag product p25 noncovalently associates with the viral genome to form a nucleoid shell (Vigne *et al.* 1982, Petursson 1990). pol

encodes enzymatic proteins reverse transcriptase (RT), protease and integrase. There is a 150 kDa (Pr150) precursor detectable in infected cells (Vigne *et al.* 1982) which is believed to be a gag and a pol polyprotein precursor. In common with other lentiviruses, only the env gene (the envelope gene) of MVV encodes the major envelope glycoprotein gp135 (the external envelope glycoprotein) and gp41 (transmembrane protein). Gp135 is involved in the recognition and binding to the target cell receptors. It has been identified as an antigenic target during immune responses to MVV infection and is probably the major viral antigen, which induces neutralising antibody in infected sheep (Scott *et al.* 1979). By analogy with HIV, gp41 appears to be a major serological marker for MVV infection (Kwang *et al.* 1992) and is involved in membrane fusion. Gp135 is noncovalently bound to gp41 and can be shed from the surface of virion.

1.1.3.2: **Accessory genes of MVV**

In addition to three structural genes, a number of regulatory genes (*tat*, *rev* and *vif*) are present in MVV genomes, which encode regulatory proteins. Although these regulatory genes have been known to have an important role in the virus life cycle, the precise roles of these genes remain ill defined. The *tat* of lentiviruses functions to activate viral gene expression at both the transcriptional and post-transcriptional level (Levy 1992). The tat gene encodes a 10 kD protein. In HIV RNAs (as in SIV, EIAV), the tat (trans-activator of transcription) protein enhances HIV genes expression by interacting with cellular factors and binding to a stem-loop structure called tat-activating region (TAR) located at 3 end of viral mRNA (Clements *et al.* of 1992). Tat may activate viral gene expression by stabilising mRNA transcripts and increasing the elongation of RNA (Oliver *et al.* 1981, Sharma *et al.* 1992). In contrast, MVV (as in CAEV) lacks a TAR element, thus the tat protein of MVV acts in a more indirect fashion (Carruth *et al.* 1994). MVV Tat protein was reported to be a transcriptional activator which does not bind directly to viral DNA or RNA. This behaviour is similar to the transactivator proteins in herpes simplex (VP16) and adenovirus (Ela) which interact with cellular factors to activate viral transcription (Smith *et al.* 1990, Horikoshi *et al.* 1991, Ingles *et al.* 1991, Roberts *et al.* 1993).

MVV Tat protein increases viral expression by interacting with the Ap-1 or AML (vis) sites in LTR, probably by interacting with cellular Jun and Fos proteins (Gdovin *et al.* 1992) or AML-1 (Sutton *et al.* 1997). The *rev* (regulator of expression of virion) gene encodes a protein (19 kD) (Mazarin *et al.* 1990) translated from multiply-spliced RNA (Sodroski *et al.* 1986). MVV Rev protein localises in the nucleolus of infected cells (Schoborg *et al.* 1994). The functional significance of Rev protein nucleolar localisation is still unclear. It is possible that the nucleolar localisation is related to association of Rev with cellular RNA and proteins and the normal trafficking pattern of those cellular components. The Rev protein functions to facilitate the export of unspliced viral mRNA from the nucleus and association of these viral RNAs with polyribosomes (Arrigo *et al.* 1991). The rev protein binds to viral RNA in the nulecus through a highly structured RNA element (the Rev-responsive elements or RRE) located within the env gene (Arrigo *et al.* 1991) and facilitates the cytoplasmic accumulation of unspliced and partially spliced viral RNA (Malim *et al.* 1989). MVV Rev was found to be present in nucleolus regardless of whether viral RNA is present in the cells (Schoborg *et al.* 1994), indicating that the nucleolar localisation is independent of binding to the RRE in the viral RNA. Consequently, Rev is required for the expression of unspliced viral RNA that encodes viral structural protein and indirectly downregulates its own expression, as well as that of tat. The role of the gene products of other MVV accessory genes in viral replication remains to be understood. The *nef* (negative factor) gene is present in all primate lentiviruses. The mechanism of action of the nef protein has not been completely defined. It has been shown that the nef protein is not essential for viral replication in cell culture (Hammers *et al.* 1989). However, SIV nef protein is required for *in vivo* efficient virus replication (Kestler *et al.* 1991). It has been reported that the expression of HIV nef protein results in reduction of CD4 expression on the cell surface (Aiken *et al.* 1994). The nef protein may, therefore, be associated with membrane structure and may function to downregulate surface expression of CD4 on infected cells, facilitating the Env protein expression without interaction with CD4. It is curious that MVV, like other strictly monocyte/macrophages tropic lentiviruses, lacks a nef gene. It is possible that

alternative gene products act as an analogue to the nef protein due to different usage of receptors. The *vif* (viral infectivity factor) gene encodes a late gene product. Vif appears to be essential for the infectivity and spread of HIV in infected cells (von Schwedler *et al.* 1993). The function of the vif protein in MVV infection is not completely understood. As the precise role of these accessory genes in virus life cycle are better defined, insights will be gained into the MVV replication process, in particular, the interaction with cellular factors.

1.1.3.3: **MVV life cycle**

The genomes of MVV are single-stranded, positive-sense RNA. The virus replication is similar to that of other lentiviruses, which involves requirement of RT enzymatic action and reverse transcription of the viral RNA to produce double-stranded DNA provirus. Once reverse transcription occurs, viral DNA can be found in the nucleus. The viral genomes are present for the lifetime of cells. Because lentiviruses use the normal mechanism of cellular protein synthesis for expression, it seems likely that, in addition to the viral regulatory proteins already described, the internal and external cellular environments can have influence on virus expression.

MVV infection is initiated by binding of viral envelope to a cellular receptor. MHC class II molecule has been identified as a potential cellular receptor for MVV (Dalziel *et al.* 1991). There may be other cell surface molecules involved in virus binding and entry, however, because some cells such as B cells and activated T cells that do express MHC class II do not confer susceptibility to MVV. In addition, cells that do not detectably express MHC class II can be infected by MVV *in vitro* (Leroux *et al.* 1995). Antibodies to an unidentified 50 kD protein from sheep choroid plexus cells can block binding of MVV to these cells in culture (Crane *et al.* 1991). Thus alternative routes of MVV entry besides MHC class II molecule may be involved. Once inside the cell, a linear dsDNA copy of the viral genome is produced by virion-associated RT. Early studies on replication of MVV *in vitro* had shown that MVV replicates productively without evidence of integration (Harris *et al.* 1984). But subsequent molecular cloning of the integrated viral DNA provided

definitive evidence that integration is an obligatory step in the MVV life cycle (List and Haase 1997).

MVV is able to replicate in non-dividing terminally differentiated cells, like other lentiviruses. This implies that cellular DNA replication may be not required for integration of the viral DNA into cellular genome, unlike other classes of retroviruses. It is still an open question whether this may require specific functions, supplied by the viral accessory genes, to permit translocation of the preintegration complex and integration into nonreplicating genomic DNA.

1.1.3.4 Restricted replication

MVV infects mainly monocyte/macrophage lineage cells (described in 1.1.4). Unlike HIV, which replicates to high titres in lymphoid tissues during the early phase of infection, MVV replicates at a low rate for an indefinite period, and is rarely present in a cell-free state in tissue during sub-clinical infection (Narayan *et al.* 1982). This type of infection with an apparent incomplete virus life cycle continues for years. A few of these infected sheep eventually develop chronic progressive disease, affecting specific tissues. In such animals, cell-free virus can usually be recovered from this affected tissue, however, an incomplete virus life cycle remains operative in macrophages from other parts of the body (Narayan *et al.* 1982). Therefore, expression of the viral RNA and viral proteins *in vivo* is restricted to a minimal rate except under certain conditions such as activation of an infected target cell when replication of virus is stimulated.

Early reports have shown that peripheral blood mononuclear cells (PBMCs) from persistently infected sheep contain incompletely expressed viral genomes. But in contrast to the *in vivo* situation, virus production was observed when these cells were cultured *in vitro* which promotes the maturation of monocytes into macrophages (Narayan *et al.* 1982, Gendelmam *et al.* 1989). The molecular mechanisms regulating this restricted replication and gene expression *in vivo* and release from the restrictions *in vitro* are poorly understood although it seem likely that interaction of

cellular factor with viral LTR may be involved in the regulation. A recent study reported that the unexpressed viral genomes were also found in maturing macrophages (Chebloune *et al.* 1996). The finding of unexpressed viral genome in MVV infected macrophages is closely consistent with the observation in HIV infected subjects who failed to develop evidence of virus replication months after viral DNA had been detected in PBMC by PCR (Horsburgh *et al.* 1989). The findings may in part explain why the actual level of detectable virus antigen is very low in MVV-infected lung (Gendelman *et al.* 1985, Lujan *et al.* 1994). Thus MVV replication either proceeded to completion, subject to the maturation of monocytes to macrophages or remained arrested at the DNA stage despite the maturation of monocytes to macrophages. Although the molecular events controlling restricted virus replication remains to be determined, it seems likely that, in addition to virus-defined restriction on MVV replication (described in 1.2.4), there is also cell-specific restriction. The cellular factors such as MVV-induced cytokines (described later in this chapter) may play an important role in restricting viral replication *in vivo* and in relieving this restriction on the viral replication *in vitro* or when a persistently infected cell is activated, possibly through interaction of certain cellular factors with the virus LTR.

1.1.4: **Pathology of MVV infection**

MVV infection in sheep causes a wide spectrum of lymphoproliferative and inflammatory lesions. Respiratory disease (Maedi) including lymphoid interstitial pneumonia (LIP) and pulmonary lymphoid hyperplasia (Narayan *et al.* 1985, Lairmore *et al.* 1986, Brodie *et al.* 1993, Watt *et al.* 1994), nervous system disease and wasting (Visna) including lymphocytic encephalomyelitis (Narayan *et al.* 1985, Nathanson *et al.* 1985, Georgsson *et al.* 1989, Watt *et al.* 1994). lymphocytic indurative mastitis (Anderson *et al.* 1985) and non-suppurative lymphocytic polyarthritis (Narayan *et al.* 1985) were reported to be infectious sequelae.Maedi is a classic interstitial pneumonia. Grossly, affected lungs show extensive consolidation and do not collapse when the thoracic cavity is opened. The lungs may be enlarged in size 2 to 4 times heavier than normal with decreased elasticity (Sigurdsson *et al.*

1954). Histologically, lesions consist of widespread infiltration of mononuclear cells into the interstitial spaces of the lung and around bronchi. The infiltration of lymphocytes, monocytes and the formation of lymphoid nodules with germinal centres in the alveolar parenchyma and around blood vessels and airways leads to a thickening of alveolar septa (Georgsson & Palsson 1971). The lung of most infected sheep shows widespread hyperplasia of smooth muscle (Watt *et al.* 1992). The regional lymph nodes draining the lung become enlarged consistently with very prominent cortices (Watt *et al.* 1994). Macrophages are activated and expressed MHC class II. Diffuse proliferation of mesenchymal cells including smooth muscle fibres, accompanies the inflammatory process (Lujan *et al.* 1994). Lymph nodes draining the lung undergo massive hyperlasia.

MVV infection of CNS (visna) induces demyelination and destruction of white matter in the cerebellum and spinal cord (Sigurdsson *et al.* 1957). Histologically, visna is characterised by chronic-active meningoencephalomyelitis and choroiditis with massive infiltrations of mononuclear cells (mainly lymphocytes with some plasma cells and macrophages) around blood vessels in the neuropil, development of microgial nodules in which macrophages are activated and expressed MHC class II, and generalised astrogliosis (Watt *et al.* 1994). There is perivascular cuffing, neuronal necrosis, malacia, and demyelination scattered patchily throughout the CNS (Watt *et al.* 1994).

In the mammary gland, infection induces chronic indurative mastitis (Van der Molen *et al.* 1985) which is characterised by marked lymphoid hyperplasia and fibrosis as observed in the affected lung. Non-suppurative lymphocytic polyarthritis/synovitis has been observed in most MVV infected sheep (Oliver *et al.* 1981) with swelling and calcification of soft tissue, fibrosis of the joint capsule and synovium and perivascular lymphocytic infiltration which is similar to CAEV induced arthritis in the goat (Wilkerson *et al.* 1995).

1.1.5: Immunopathology of MVV infection

There is now evidence suggesting that an important event in the initial establishment of lentivirus infection is localisation of the viruses in lymphoid organs that serve as major reservoir and as primary sites for infection (Bird *et al.*1993, Pantaleo *et al.* 1993). Bird *et al.* (1993) performed lymphatic cannulation on sheep to monitor virus at different-time points after infection. Detection of MVV in lymph nodes was observed as early as day 9. At this time, numerous individual cells expressing MVV were detected in efferent lymph, which is consistent with the study of Pantaleo *et al.* (1993) showing SIV replication was observed in lymph node biopsies as early as day 7 post-infection. These results demonstrate that MVV, SIV and likely HIV, localise very early in lymphoid organs and support the concept that lymphoid tissues may represent a primary sites for the initial establishment of infection and be important in determining viral persistence. However, it dose not exclude the possibility that other anatomical sites may be of importance in pathogenesis. Although sheep mount an anti-viral immune response, viral replication and dissemination occur (Bird *et al.* 1993). Therefore, the immune response during MVV infection is more complex and more ambiguous. As has been demonstrated in HIV, the ability of MVV to replicate depends in part upon the state of the target cells. Different types of effector cells become activated during the generation of a specific immune response. This process of activation may have a stimulating effect on MVV infection by way of generation of activated target cells for MVV replication and production of cytokines which may exert stimulating effects on MVV infection such as induction of viral expression or modulation of certain viral component synthesis in infected cells (Poli *et al.* 1992a, Del Prete *et al.* 1993, Ellis *et al.* 1994).

The interaction of MVV with the immune system may contribute to the propagation of MVV infection and the appearance of certain immunopathological lesions during the course of MVV infection. Induction of cytokine expression in the site of disease in particular merits consideration but the precise roles of MVV and immune effector cells which induce chronic inflammation remain to be determined. Interaction of T cells with infected macrophages has been shown to result in the production of certain

cytokines with interferon-like activity (Lairmore *et al.* 1988a). This interferon-like molecule was discovered in cell culture, but has also been seen in synovial fluid of arthritis in CAEV-infected goats (Kennedy-Stoskopf *et al.* 1989) and in culture supernatants of lymphocytes from the lung of MVV-infected sheep (Lairmore *et al.* 1988a). It is not known whether the release of this cytokine had a role in the pathogenesis of the lesions. However, secretion of the cytokine may explain the activation of macrophages as well as the occurrence of free virus in the affected sites. Lymphocytes from these lesions failed to produce virus, whereas macrophages cultured from the affected tissues was able to produce cell-free virus. Thus, productive virus replication in local tissue macrophages contributed to lesions *in vivo*, pneumonia being associated with virus production in alveolar macrophages and encephalitis lesions associated with virus production in microglia.

The presence of high level of antibodies to viral glycoprotein at the sites is another feature of the lentiviruses infection-related lesions. A large number of plasma cells were observed in progressive pneumonia (Kennedy *et al.* 1985) and the antibody to viral glycoprotein was found in CAEV related lesions (Johnson *et al.* 1983). Studies by Nathanson *et al.* (1981) and McGuire *et al.* (1986) extended these observations, showing that immunisation of infected sheep and goats with the viral antigens led to an increased severity of lesions. These studies suggest that immunoglobins could be produced locally at these sites and play a critical role in the pathogenesis of the lesions. Taken together, the immunopathological characteristic of MVV-induced local tissue lesions is productive virus replication in macrophages, activated macrophages, release of cytokines and high level of anti-viral antibodies. All of these factors are also observed in HIV-induced lesions.

1.1.6: **Identification of MVV infection**

Infection after exposure to MVV is not inevitable. Following viral entry, there is an initial period of virus replication during which antibody is undetectable, i.e. detection of antibodies specific for a variety of MVV proteins. In addition, in a small minority of animals, the seronegative phase may persist for months. Therefore the

identification of virological, immunological and clinical events at this stage of infection are difficult and their diagnosis relies upon the detection of viral genome.

1.1.6.1: **Detection of antibody**

Antibody can be detected by a number of methods including the Agar gel immuno-diffusion (AGID) test, ELISA and Western Blot. The AGID test for detection of group-specific antibody to MVV first described by Cutlip *et al.* (1977) has been used widely. It is based on the availability of a semi-purified soluble antigen prepared from MVV infected cells and a positive control serum produced by hyper-immunisation of sheep. Although the AGID test is simple to perform and rapid, it is not highly sensitive and quantitative (Houwers *et al.* 1989, Zanoni *et al.* 1989, Torfason *et al.* 1992). This lack of sensitivity is a consequence of poorly defined viral antigens and reagents that contains cellular proteins from the tissue culture in conjunction with the inherent insensitivity of the AGID test compared with that of other serological tests. Generally, MVV antibodies as measured by the AGID test appear at the third week after infection when inoculated by the intratrachael route (Simard *et al.* 1990). The AGID test may not be adequent to monitor samples for an eradication scheme.

Several ELISA assays have been evaluated as alternatives to the AGID. Initially indirect (I) ELISA with higher sensitivity, but modest specificity due to the use of semi-purified antigen prepared from the infected cells was described (Houwers *et al.* 1982). In order to reduce the amount of cellular proteins interfering with the test and to increase the specificity of I-ELISA, Vitu *et al.* (1982) used sucrose density purified the viral antigen to obtain good discrimination between the positive and negative serum samples. Later, Simard *et al.* (1990a, 1990b) applied an anionic detergent SDS for preparation of MVV antigen to obtain an ELISA with a specificity of at least 98.8 % and an increased relative sensitivity of 15.5% compared to the AGID test and with an average ability to detect MVV antibodies 2-6 weeks prior to the AGID test. In 1987, Houwers *et al.* (1987) described a complex trapping blocking ELISA (CIB-ELISA) in which two monoclonal antibodies with different

epitopes specificity were involved in a modified double antibody sandwich blocking procedure. CIB-ELISA was found to be more sensitive than I-ELISA and AGID for detecting anti-bodies to MVV. Unlike the AGID test, CIB-ELISA was highly specific and resulted in low false reaction due to two epitopes instead of one, and resulted in a widening of the antigenic window.

Recently, recombinant gene expression products have been used and well documented in anti-viral antibody detection. Zanoni *et al.* (1991) reported that development and application of gag-derived recombinant antigen in I-ELISA for the detection of antibodies to ovine and caprine lentiviruses. It was found that the recombinant antigen was superior to antigen prepared from the whole virus antigen. A similar recombinant ELISA was also described by Kwang *et al.* (1992) who developed and used a recombinant protein corresponding to the NH2-terminal hydrophilic region of gp41 of MVV in I-ELISA. It was shown that a single recombinant gp41 can be used successfully for the generation of specific ELISA for the detection of most MVV infected animals, which suggested that like HIV, gp41 appeared to be a major serological marker for MVV infection. The oligopetides-based ELISA where the synthesised oligopetides represent regions encoded by the portion of the env gene proved it further. However, a single recombinant protein in ELISA may cause a false negative due to an individual sheep lacking antibody to that antigen of MVV. Therefore to compensate for the loss of antibody reactivity with one gene derived protein during the course of infection or early stages of infection, as seen often in HIV infection, so the use of more than one recombinant proteins in ELISA may be necessary for the detection of all potentially infected animals. Western blot allows the detection of antibody to specific viral proteins and it therefore is widely used to analyse the antibody response to MVV infection (Houwers *et al.* 1989, Zanoni *et al.* 1989, Torfason *et al.* 1992). In MVV infection, Western blot demonstrated that, generally, antibodies against Gag proteins and the major env glycoprotein appeared early while the response to gp41 appeared later. However, these were found in specimens with no antibodies to gp135 (Houwers *et al.* 1989). The possible reason for this is that the Western blot is less sensitive in

detecting glycosylated proteins. This has been also found in HIV (Saah *et al.* 1987). Due to loss of polysaccharides during preparation, this has led to some early difficulties in the diagnosis of MVV infection, so further refinement of MVV Western blot assay may solve this problem (Torfason *et al.* 1992). The results of Western blotting showed the persistence and predominance of p25 antibodies in serum of infected sheep, which were strongly supported by the p25 specific ELISA (Houwers and Schaake 1987). The analysis of antibody recognition patterns in sheep with or without lesions demonstrated a reduction in antibody during advanced stages of clinical disease (Torfason *et al.* 1992). The cause of this declining antibody activity remains to be elucidated. One possible explanation of this phenomenon is that at the advanced stage of disease the animal had high titres of cell-free virus and that causes the formation of antibody-viral antigen complexes.

As noted above, the use of recombinant antigens has produced assays of greater specificity. However, it is clear that the assay with narrow specificity may give a false negative result. The ELISA employing recombinant gp41 failed to identify sheep at an early stage of infection although increased specificity was produced compared with the AGID test (Kwang 1994). Consequently it is necessary to use more than one recombinant protein antigens in ELISA and Western blot to detect all the potentially infected sheep. Furthermore, at the early stage of infection, immune responses to virus may either be absent or too weak to be detected. Thus it is for this reason that inconlusive sera must be further investigated carefully.

1.1.6.2: **MVV antigen detection**

ELISA is presently the predominant antigen assay for MVV used for clinical investigation (Marcom *et al.* 1992, Brodie *et al.* 1995). The MVV p25, which occurs in relatively high abundance in virions and infected cells (Reyburn *et al.* 1992), is generally the target of this assay. By using this assay, as little as 3.2ng of viral p25 antigen per ml can be detected (Marcom*et al.* 1992). Immunohistochemical assay is also useful as a means of detecting MVV antigens in tissue section or cells. By use of this method, MVV antigens could be detected in cells morphologically and

histochemically compatible with cells of macrophage lineage which are usually located within or adjacent to areas of lymphocyte aggregation or lymphoid proliferation in brain, cervical spinal, cord, distal trachea, lung, mammary gland, spleen, synovium and node associated with drainage of such tissue (Brodie *et al.* 1995).

1.1.6.3: **MVV nucleic acid detection method: Polymerase Chain Reaction (PCR)**
The diagnosis of the MVV infection has routinely relied on demonstrating the presence of MVV antibody (Houwers *et al.* 1982, 1989, Simard *et al.* 1990, Zanoni *et al.* 1989, 1991a, Kwang *et al.* 1992, Torfeson *et al.* 1992). However, these antibody assays do not detect virus during the period between infection and seroconversion and do not give a quantitative measurement of virus load in infected hosts. They are therefore not useful to study the initial events following viral infection, the relationship between virus load and pathogenesis and the dynamics of viral infection.

Recently, the PCR has been used extensively in the study of MVV, especially in rapid diagnosis and pathogenesis (Haase *et al.* 1990, Zanino *et al.* 1991, 1992, Johnson *et al.* 1992, Reyburn *et al.* 1992, Ding and Xiang 1994, Woodall *et al.*1994a). The PCR can amplify genetic material from a single cell or tissue samples by approximately 106 fold in a matter of hours and it is more rapid, sensitive and less labour intensive than the nucleic acid hybridisation and virus isolation for detection and identification of MVV. Because of this extreme sensitivity, the PCR is able to detect the MVV at the time of the early infection "Diagnostic Window" or during periods of viral latency (Johnson *et al.* 1992). This concept is further supported by a recent study that the presence of MVV DNA was detected in PBMC from infected sheep but neither viral particle nor protein could be detected in some of these cells (Chebloune *et al.* 1996) although the significance of PCR positive in seronegative individuals remains to be further elucidated.

In PCR assays, it is important and necessary that the methods used to isolate nucleic

acid be as simple and efficient as possible. PBMCs are the cells most often used for PCR detection of MVV. Sometimes, the cells are treated with stimulators of RNA expression to decrease false negative in PCR. For example, low viral burden in cells is main cause of failure for detection of HIV by PCR (Zazzi *et al.* 1995).

PCR for MVV is performed mainly on cDNA (RT-PCR). Compared to HIV, there are fewer PCR primer sets published for MVV. Zanoni *et al.* (1992) used a set of three primer pairs derived from MVV gag, LTR and pol genes for the amplification of the corresponding sequences of six virus isolates. Only with LTR derived primers, could the corresponding sequences for all strains be detected. These primer sequences differ slightly simply due to inter-strain variability, which is usually not a problem as long as the homology remains sufficiently high to allow specific hybridisation. Currently, PCR has been used to detect MVV RNA directly in clinical specimens (Brodie *et al.* 1993) and infected cell culture (Johnson *et al.* 1992, Zanoni *et al.* 1992). Hasse *et al.* (1990) developed a PCR combined with in situ hybridisation and applied it successfully for detection of MVV DNA inside experimentally infected cells. The above report and others hold promise for rapid detection of MVV RNA, but standardisation of PCR procedures and extensive validation with field samples are required before routine application for diagnosis.

Quantitative competitive PCR (QC-PCR) has been developed to investigate the absolute quantitation of viral nucleic acid in order to address fundamental virological and molecular aspects in pathobiology of viral infection (Menzo *et al.* 1992, Pistello *et al.* 1994, Zhu *et al.* 1995). QC-PCR has been shown to be a very sensitive and reliable method of measuring the viral load in infected hosts, and in general, detection of viral nucleic acid has become a valuable diagnostic tool. Although PCR for detecting MVV nucleic acid was reported (Haase *et al.* 1990, Zanoni *et al.* 1992, Reyburn *et al.* 1992, Johnson *et al.* 1992, Brodie *et al.* 1993, Ding and Xiang 1994), it has been mainly limited to qualitative analysis.

1.2: **Cell biology of MVV infection**

1.2.1: **Cell tropism of MVV**

MVV infects a restricted range of target cells *in vivo*, principally, monocyte/ macrophages (Narayan *et al.* 1990). MVV cell tropism may contribute in part to the capacity of the virus to persist in its host. But multiorgan disease observed during MVV infection can not be explained solely by the interaction between lentiviruses and its target cells. Other cells have been directly or indirectly shown to be infected. Cell studies have shown that the content of infection varies and can depend on the particular virus strain used. Generally, highly cytopathic stain replicates to a higher titre (Marcom *et al.* 1992).

Monocyte/macrophages are the principal cells for MVV infection *in vitro* and *in vivo*. *In vitro*, MVV can be grown productively and induces a cytopathic effect with the formation of syncytia in monocytes/macrophages. *In vivo*, the MVV-infected monocytes/macrophages are found in peripheral blood, spleen, lung, brain, spinal cord, lymphoid node, mammary gland, bone narrow, synovium (Narayan *et al.* 1982, 1985, Gendelman *et al.* 1985, 1986, Gorrel *et al.* 1992, Brodie *et al.* 1995). In certain tissues, notably lung and lymphoid node, the detection of viral proteins or infectious virus in infected target cells is associated with lymphocyte infiltration (Brodie *et al.* 1995). Although MVV RNA was detected in a variety of target and non-target tissues and in several morphologically distinct cells types including monocytes, macrophages, epithelial cells and in tissues with and without MVV-associated lesions, only in cells of the macrophage lineage can the MVV capsid proteins be detected, usually in inflamed tissues or only in target tissues (Brodie *et al.* 1995). In infected animals, MVV infection of mononuclear phagocytes occurs predominantly in terminally differentiated macrophage in tissues such as the lungs and brain. Alveolar macrophages (AM) bearing MVV capsid protein has been found at the periphery of such lesions (Brodie *et al.* 1995), estimates of the proportion of infected alveolar macrophages range from less than 1% to 12% (Luján *et al.* 1994) as measured by immunohistochemistry. In brain, resident macrophages have been showed the presence of MVV (Georgsson *et al.* 1989). Although productive

infection is found in macrophages, macrophages in some tissues such as Kupffer cells in liver are not infected with MVV (Brodie *et al.* 1995). This selectivity of different macrophage populations for preferentially high level of virus replication forms the basis of localisation of histological lesions in tissues. It is unclear what factor or factors allow productive infection of some macrophages but not others although it may be explained in part by a lack of receptors for the virus because MVV infection is dependent on virus binding to specific receptors (Gendelman *et al.* 1986, Kennedy-Stoskopf 1989). Such factors potentially play a critical role on the tissue distribution of lentivirus and thus potentially in its clinical manifestation.

MVV-infected macrophage precursor cells are present in bone marrow of MVV infected animals (Gendelman *et al.* 1985). As macrophages are terminally differentiated cells with relatively a short life-span, it is likely that these macrophage precursor cells (long life-span) in bone marrow where infection becomes established early after infection, act as a viral reservoir for dissemination of MVV.

MVV infects monocytes but viral replication is highly restricted in these cells (Narayan *et al.* 1982, Brahic *et al.* 1981). It is clearly known that susceptibility of cells to infection as well as the magnitude and rate of viral RNA transcription and protein synthesis is closely linked to the rate of maturation of monocytes to macrophages, i.e. the restriction of maturation of the infected cells limits the replication of virus (Narayan *et al.* 1985). However, mechanism for the behaviour is not yet clear. Macrophages play an important role in MVV disease. It has been suggested that the replication of MVV macrophages in the lung produces a cascade of molecular and immunological events that lead to chronic inflammatory lesions (Narayan *et al.* 1989). The persistent expression of viral antigens on the surface of these infected cells may be of considerable importance in facilitating these lesions.

In addition, MVV has been detected *in vivo* in oligodendrocytes, astrocytes and replicates *in vitro* fibroblasts (Georgsson *et al.* 1989). It is unclear whether MVV may infect and replicate in CD4+ T lymphocytes, as occurs in HIV and SIV (Levy

1993), although it has been demonstrated that the proportion of CD4+ T lymphocytes decrease during MVV infection in lungs (Luján *et al.* 1993). Early studies suggested MVV infection in lymphocytes within the CNS, in glial cells and in the bronchiolar epithelial cells in the lung (Stowring *et al.* 1985, Georgsson *et al.* 1989, Staskus *et al.* 1991). However, these reports have not been supported by other published data, which failed to find viral replication in lymphocytes from blood, lymph and lung (Gorrel *et al.* 1992, Luján *et al.* 1994). Lack of productive infection in lymphocytes may be due to the absence of receptor for MVV.

Gorrell *et al.* (1992) showed that MVV has a greater tropism for dendritic cells than monocytes, which is consistent with the previous observation that MVV infection is greater in lymphoid organs than in liver (Narayan *et al.* 1985). MVV Infection of ovine smooth muscle cells *in vitro* has been demonstrated recently (Leroux *et al.* 1995), suggesting that the infected smooth muscle cells may act as another reservoir for virus in addition to myeloid stem cells in bone marrow. This finding probably helps to explain the induction of pathogenic reaction like myomatosis in the lung of MVV infected sheep (Georgsson & Palsson 1971). There is a recent report that human cytomegalovirus stimulates p53 accumulation and that the IE84 CMV proteins blocks the p53's inhibition of cell cycle progression (Speir *et al.* 1994). A similar mechanism could occur during MVV infection. The interaction between MVV regulatory proteins and cellular genes induces a smooth muscle cell proliferation.

1.2.2: **Phenotypic characteristic of MVV-infected monocytes/macrophages**

The expression of surface molecules on monocyte/macrophages is often used as an indicator of their functional activity. Studies of the expression of the cell surface molecules on monocytes/macrophages infected with MVV showed the expression of the cell surface molecules could be affected during the infection (Luján *et al.* 1993, 1994, Lee *et al.* 1996). It had been shown that the AMs of MVV-infected sheep showed increased expression of major histocompatibility complex (MHC) class II (Luján *et al.* 1993). The same phenomenon was also observed on macrophages from

synovial fluid of MVV infected sheep (Harkiss *et al.* 1991). There is no difference in the expression of LCA between the AMs from MVV-infected sheep and control animals. AMs of both animals lack the expression of CD4 and CD8 (Luján *et al.* 1993). However, The expression of CD4 and CD8 was seen on blood monocyte-derived macrophages from both MVV-infected and control animals (Lee *et al.* 1996). Lymphocytes from bronchoalveolar lavage fluid (BALF) of MVV-infected animals showed increased expression of LFA-1 and LFA-3 compared to that of control animals (Luján *et al.* 1993). In HIV infection, the expression of the cell surface molecules on macrophages has been studied in more depth (Wright *et al.* 1988, Rossen *et al.* 1989, Vermot-Desroches *et al.* 1990, Pantaleo *et al.* 1991, Fecondo *et al.* 1993, Berman *et al.* 1994a, Stent *et al.* 1994). The AMs from HIV-infected individuals with lymphocytic alveolitis showed elevated MHC class II antigens HLA-DR, -DQ and -DP expression (Agostini *et al.* 1993a). This increase may account for the increase of antigen presenting capacity of AMs from HIV infected individuals, enabling T cells to recognise alloantigens better (Twigg *et al.* 1989). AMs from HIV infected individuals also show high-affinity receptors for the Fc portion of IgG (CD64), IgE (CD23) and complement (CD11b) and increased expression of other activation markers (CD11c, CD14 and CD71) and adhesion molecules (CD11a/CD18, CD44, CD54) (Agostini *et al.* 1993a). A correlation between histological evidence of AIDS encephalitis and increased expression of VCAM-1 on parenchmal vessels was observed in the brain tissue of experimentally infected animals with SIVmac (Sasseville *et al.* 1992).

The lesions caused by MVV comprise chronic active inflammatory changes with lymphocyte infiltration and proliferation (Lairmore *et al.* 1986, 1988b, Watt *et al.* 1994). Thus alterations in the level of the expression of the surface molecules on macrophages as a result of infection could play an important role in the pathogenesis of the lymphoproliferative response and viral persistence. The increased expression of the MHC class II molecule, a possible component of cellular receptor for MVV (Dalziel *et al.* 1991), may favour the spread of the infection to uninfected macrophages, allowing the virus persistence within the host. The persistent

expression of these surface molecules may contribute to the accelerated entry and accumulation of lymphocytes into the lung interstitium as the disease progresses, mediating local lesion. Furthermore, activation of T lymphocytes (Watt *et al.* 1992) and the increased expression of LFA-1 and LFA-3 as well as MHC class II molecules on the T lymphocytes from MVV infected sheep (Luján *et al.* 1993) was reported. Because LFA-1 and LFA-3 is essential for leukocytes activation and trafficking (Pardi *et al.* 1992), the increased expression of these molecules could contribute to lymphoproliferative response by promoting constant lymphocyte recruitment into the lung. However, the exact role of these surface molecules in pathogenesis of lentiviral infection remains to be determined.

Virus regulatory mechanisms responsible for modulation of cell surface molecules of infected monocytes/macrophages are not fully understood. It has been hypothesised that the proportion of MVV-infected monocytes in the blood is low (McElrath *et al.* 1989), therefore it is unlikely that changes in the expression of cell surface molecules are in part directly related to viral infection of the cells. Increased MHC class II molecule was expressed on MVV-infected AMs and co-expressed with viral protein, but, no direct relationship was observed between viral production and the MHC class II expression (Luján *et al.* 1994). Thus mechanisms responsible for modulation of cell surface molecules on infected monocyte/macrophages may be that the virus stimulates cells to release various cytokines and other factors which may in turn alter surface molecule expression. The interaction of lymphocytes with MVV-infected macrophages leads to the release of lentivirus IFN (Narayan *et al.* 1985) and it may be through the action of this cytokine that the expression of surface molecules is increased. Increased expression of MHC class II molecules but decreased CD5 expression (Luján *et al.* 1993) on T lymphocytes suggest that they are activated and may be producing cytokines which in turn alter the surface molecule expression on macrophages. Several studies have shown that HIV, by stimulating the production of IL-1 and TNF, leads to autocrine feedback with production of other cytokines such as IL-6 and GM-CSF (Merrill *et al.* 1991, Longo *et al.* 1993). In *in vitro* cell systems, cytokines or combination of cytokines have

been reported to modulate expression of antigen presenting (MHC class I and class II) and co-stimulatory molecules on the surface of monocytes and other leukocytes (Mentzer *et al.* 1986, Springer *et al.* 1986, Freyer *et al* 1988, Te Velde *et al.* 1988, Jiang *et al.* 1992, Nash *et al.* 1992, Gimcher *et al.* 1992, Schwartz 1992, Sallusto *et al.* 1994, Kruger *et al.* 1996, Thomssen *et al.* 1996, Lee *et al.* 1997). GM-CSF down-regulates CD14 expression (Kruger *et al.* 1996) and induces expression of CD1a, b, c molecules (Kasinrerk *et al.* 1993) on monocytes. IFN increased surface expression of MHC class II molecules (Nash *et al.* 1992), but TGF-β suppresses IFN-γ induction of MHC class II gene expression by inhibiting accumulation of class II transactivator mRNA (Lee *et al.* 1997). IL-3 in combination with IL-4, induces expression of CD1 on monocytes and this induction of CD1 by IL-3 can be suppressed by IL-10 (Thomssen *et al.* 1996). Thus it is likely that changes on expression of cell surface molecules on monocytes/macrophages are in part directly related to cytokines. However, monocytes treated with purified gp120 *in vitro* show increased MHC class II expression (Wahl *et al.* 1989a, Allen *et al.* 1990). Thus only future studies will elucidate this question.

1.2.3: **Functional changes in infected monocytes/macrophages**

Monocytes/macrophages are capable of eliminating intracellular organisms. Nevertheless, these cells are excellent reservoirs of MVV infection (Brodie *et al.* 1995). It is still an open question about the direct effect of MVV infection on the function of these cells and little is known about their function as effector cells against MVV. In early studies, a decrease in the delayed type hypersensitivity response to tuberculin was observed in MVV-infected sheep (Myer *et al.* 1988), suggesting that MVV infection could cause macrophage function to be impaired *in vivo*. AMs from MVV-infected sheep with interstitial pneumonia showed a significant increase in fibronectin and neutrophil chemotactic factor release (Cordier *et al.* 1990), but not the expected increase in phagocytosis considering the activation phenotype of the cells (Lee *et al.* 1996). AMs from MVV infected sheep were reported to show normal Fc-receptor-mediated (immune system-specific) phagocytosis (Lee *et al.* 1996) and monocytes from MVV-infected animals also

showed no defect in the binding and phagocytic activity for bacteria (Lee *et al.* 1996). Similar phenomenon also occurs in HIV infection. Monocytes from HIV-infected individuals showed normal C3-receptor-mediated (nonspecific) or Fc-receptor-mediated (immune system-specific) phagocytosis (Gersoft *et al.* 1982, Estevez *et al.* 1986, Nielsen *et al.* 1986, Roux-lombard *et al.* 1986, Bender *et al.* 1988), and normal lytic activity (intracellular killing) (Murray *et al.* 1985, Nielsen *et al.* 1985, Müller *et al.* 1990, Bravo-Cuellar *et al.* 1992). *In vitro* infected monocyte-derived macrophages (MDMs) show defective phayocytic activity but the normal expression of the surface molecules (Lee *et al.* 1996), suggesting that the phenotypic alterations observed *in vivo* (see 1.2.1) may be not directly mediated by MVV but by inflammatory interactions at local lesions probably involving cytokines. However, defective chemotaxis also occurred in monocytes from healthy individuals after *in vitro* exposure to HIV gp41 or gp120 (Wahl *et al.* 1989a).

Monocytes/macrophages are capable of presenting antigen in the context of an MHC molecule (accessory function). For CD4+ lymphocyte activation, it is required that antigen be presented in the context of a MHC class II on the surface of macrophages. HIV chronically infected myeloid cells with normal or decreased MHC class II expression (Roy *et al.* 1987, Melendez-Guerro *et al.* 1992) showed a decreased ability to stimulate T cells (Foely *et al.* 1992). A Similar phenomenon may occur in MVV infection because MVV is genetically and pathogenically similar to HIV. Infection of monocyte/macrophage may also lead to dysfunction in the secretion of cytokines which may responsible for the persistent of MVV in these cells.

Study of the state of activation of alveolar lymphocytes from MVV infected sheep shows an anomalous pattern of T cell activation (Begara *et al.* 1995). While T cells from MVV-infected sheep showed increased expression of MHC class II, IL-2 receptor (IL-2R) is lacking, suggesting an impaired alveolar T cells activation during the course of MVV infection. The decreased response to activation seen in alveolar T cells from MVV-infected sheep (Begara *et al.* 1995) could play an important role in the pathogenesis of MVV infection.

1.2.4: **Virus replication in monocytes/macrophages**

MVV replication in monocyte/macrophages is not well understood. In general, these cells do not divide (Metcalf 1989) but are permissive for MVV replication *in vitro* (Gendelman *et al.* 1986, Georgsson *et al.*1992). These cells differ in susceptibility to MVV infection. It is unclear whether the virus maintained in them is in certain form of latent state. A recent study showed that blood monocytes are resistant to productive HIV infection *in vitro* immediately after isolation, and further investigation found that replication of HIV was blocked prior to reverse transcription in fresh isolated monocytes (Sonza *et al.* 1996), but whether this block is at the level of binding or entry or following entry remains to be determined. It is not unclear whether the similar phenomenon occurs in MVV infection of monocytes. But the question raised is why the viral replication is blocked in these cells (Sonza *et al.* 1996). It has been shown that MVV infection of monocytes *in vitro* appears to be linked to differentiation, and susceptibility of cells to infection as well as the magnitude and rate of viral RNA transcription and protein synthesis is closely linked to the rate of maturation of monocytes to macrophages (Brahic *et al.* 1981, Narayan *et al.* 1982, 1985, Harper *et al.* 1986, Gendelman *et al.* 1989, 1992, Rich *et al.* 1992). However, the mechanism for this restriction in monocytes and how this restriction changes with maturation process remain unclear. A recent study reported that that ability to produce IFN-α, an anti-viral cytokine, is lost in response to HIV-1 as monocytes differentiate into macrophages (Francis *et al.* 1996). This finding may in part explain that why macrophages are more susceptible to infection. Furthermore, low levels of IFNs (β and γ) and/or other negative factors may be induced in monocytes in response to lentiviral infection (Gendelman *et al.* 1992, Gessani *et al.* 1994, Francis *et al.* 1996). A study extended this hypothesis showing that adherent cells of PBMCs can produce significantly higher levels of TNF-α than in non-adherent cells when stimulated with Sendai virus (Aderka *et al.* 1986). IFN-β is very effective in inhibiting viral replication in differentiated macrophages but not in freshly isolated monocytes (Gessani *et al.* 1994). Moreover, macrophages responded more effectively than monocytes to the priming effect of IFN-β for IL-12 production (Gessani *et al.* 1997). Thus the difference in response to antigenic stimulation

between monocytes and macrophages may also play an important role in the different susceptibility to MVV within these cells.

Although productive infection of MVV is often found in macrophages, little is known about the characteristics of macrophage infection by MVV *in vivo*. HIV-1 replication in AMs *in vivo* in maintained in a latent proviral form without viral replication, indicating that AMs are latently infected by HIV *in vivo* but are not a site for viral replication. In contrast, HIV-1 replication occurs with stimulation *in vitro* (Lebargy *et al.* 1994). Since the pathology of MVV-induced lesions in lung resembles that of HIV, a similar phenomenon may also occur in MVV infected AMs *in vivo*. This could be related to a still unknown mechanism that inhibits replication of virus in AMs *in vivo*. Such an inhibition could be related to various cytokines released by leukocytes present in the lung of infected subjects, such as IFN-γ. It has been demonstrated that IFNs are present in culture fluids from AMs from lambs experimentally infected with MVV (Lairmore *et al.*, 1988a) and *in vitro* interaction between lymphocytes and MVV infected macrophages restricted replication of the virus in cultured macrophages (Moench *et al.*, 1985). The IFN present in culture fluids from infected animals with lymphocytic interstitial pneumonia showed to have moderate inhibitory effects on MVV replication *in vitro* (Lairmore *et al.* 1988a). Since cytokines may be produced and may act locally in tissues without entering the circulation, their plasma concentration need not be elevated for them to have an important role in the modulation of the virus expression at tissue level. This mechanism could be a major negative control of replication in the lung, limiting the spreading infection. However, there are many other factors involved either directly or indirectly in regulating virus replication in macrophages *in vivo* that await elucidation.

1.3: **The role of cytokines in lentiviral infection**

1.3.1: **Cytokines**

Cytokines are consisted of a group of secreted regulatory proteins which act as intercellular signals in a variety of cellular response including the immune response

(Paul and Seder 1994). The molecular mass of cytokines ranges between 6 and 70kD, and often contain protective carbohydrates and intramolecular disulphide bonds. Cytokines are secreted by a variety of cell types such as macrophages and lymphocytes. Constitutive production of cytokines is usually low or absent and further expressed to varying degree in immune response to antigen (Paul and Seder 1994). A single cytokine often may have multiple target cells and may both stimulate and inhibit the production of others (pleiotropy). On the other hand, structurally dissimilar cytokines may have a similar spectrum of actions (redundancy). This implies that the loss of one cytokine activity may be compensated by that of others, and it stresses the vital importance of these interactions.

The pathological manifestation of lentirviral infection is the result of complex interactions between the direct cytopathic effect of viral infection and local and system immune response. It is now clear that the immune system is regulated by a complex network of redundant and pleiotropic cytokines. Cytokines play an important functional role in maintaining normal immune homeostasis (Paul and Seder 1994). In addition, cytokines are essential for the interaction between effector and accessory cells during host defence mechanism in response to infectious agents and for lymphocyte activation, antibody production and cytotoxic cellular responses. It is also clear that cytokines secreted either directly by infected cells or indirectly by lymphocytes and macrophages activated by the immune response to viral proteins have the key role in the pathological consequences of viral infection and its virulence (Mosmann and Coffman 1991). Lentivirus infection leads to the development of antiviral immune responses (Narayan *et al.* 1985). However, the role played by the immune response during lentiviruses is not restricted to this anti-viral immune response. Lentiviruses persistently replicate within cells of the immune system. This is a persistent source of immune cell activation. This activation is intimately linked to the expression and production of cytokines (Paul and Seder 1994). Taken together, it has been suggested that cytokines have a variety of roles during lentiviruse infection, some of which will benefit the host and others the virus. Some cytokines may contribute to stimulate or inhibit viral replication, but may also

be in involved in the development of pathological manifestation of the infection. Therefore, characterisation of the production and the role of cytokines, although difficult, would be very valuable for understanding that pathogenesis of the viral infection.

1.3.2: **The role of cytokines in modulation of the immune response**

Cytokines represent major factors in the development of immune responses. The different cytokines produced by different antigen-presenting cells and other cell types in response to infection have an important influence on the type of immune response that is induced. On the basis of the pattern of cytokines expressed, immune response can be divided into Th1 and Th2. Th1 cytokines are IFN-γ, IL-2, TNF-α/β produced by Th1 cells which promote cell-mediated immunity and delayed-type hyper-sensitivity (DTH) (Mosmann *et al.* 1986, Romagnani 1994, Mosmann and Coffman 1991). Th2 cytokines are IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 secreted by Th2 cells which favour allergic reactions and provide the most efficient help for B cells (antibody production) (Mosmann *et al.* 1986, Romagnani 1994). IL-12, secreted by macrophages and B cells, has been shown to act directly on CD4+ naive T cells derived from T receptor transgenic mice to induce IFN-γ, production and T cell differentiation towards Th1 development (Seder *et al.* 1993). In contrast, IL-4 has been shown to drive Th2 cell development and the expression of Th2 cytokines. Moreover, Th2 cytokines IL-4 and IL-10 has been shown to inhibit IL-12 production in human macrophages and thereby, the Th1 response. As both IL-10 and IL-12 are macrophage-derived cytokines, these can either stimulate or inhibit Th1 response, depending on the relative production of either cytokines by these cells. IFN-α stimulates the development of Th1 cells and blocks Th2 development (Sher *et al.* 1992). It has been reported that in certain pathologic conditions different patterns of cytokine expression may be related to different types of immune response (Mosmann *et al.* 1986, Romagnani 1994). In mice, Th1 and Th2 responses play a critical role in viral clearance (Braciale and Braciale 1994). However, In comparison with mouse and man little information is available regarding the Th1 and Th2 cytokine response in sheep and many other animal species. The existence of a dichotomy in Th1 and

TH2 cytokine response remains ill defined.

The factors responsible for initiating a switch from asymptomatic to symptomatic disease expression in lentiviral infections have yet to be determined. With regard to HIV infection, a switch from Th1 to Th2 cytokine responses has been suggested to represent a critical step in the disease based on the pattern of cytokines production from PBMCs from HIV-infected individuals at different stages of disease progression (Clerici and Shearer, 1993). Patients with Th1-type response, characterised by production of IL-2 and IFN-γ remain as asmptomatic whereas those that switch from Th1 to Th2, as indicated by decreased secretion of IL-12 and increased production of IL-4, go on to be symptomatic (Clerici and Shearer 1993, Chehimi *et al.* 1994). Further-more, recent studies demonstrate that the decreased percentage of cells expressing IL-2 and IFN-γ correlates with an increased proportion of IL-4 and IL-10-secreting cells on a single cell level among CD4+ T cells in HIV-1 infected individuals (Klein *et al.* 1997). In cats, a switch from Th1 to Th2 response has also been suggested as playing a role in FIV infection based the observation that asmptomatic cat produces high level of IL-2 (a Th1 cytokine) and low level of IL-6 (a Th2 cytokine) (Lawrence *et al.* 1995).

MVV, like other lentiviruses, persistently infects monocytes/macrophages, which leads to mononuclear infiltration of various tissues. Thus it has been proposed that a similar mechanism of immune dysfunction, involving a switch from Th1 to Th2 cytokine response may be occurring during MVV infection. In support of this, Woodall *et al.* (1997) reported that MVV-infected sheep with lung lesions showed significantly increased expression of IL-10 mRNA and elevated expression of IL-4 mRNA detected in lung tissues whereas low level of IL-10 and undetectable IL-4 mRNA expression in lung tissue of MVV infected sheep without lung lesions. Interestingly IFN-γ mRNA, marking cytotoxic T lymphocyte activity, and IL-2 receptor, marking T lymphocyte activation, was detected in the lung tissue of MVV-infected sheep with lung lesions. Very low or undetectable levels of these cytokines were found in the lung tissue of MVV-infected sheep without either lung lesions or

clinical signs. It is not clear, however, what type of cell is responsible for the expression of these cytokines. A study of cytokine expression in CAEV-induced arthritis (CAEV is closely related to MVV) showed Th1 and Th2 cytokine expression in the same arthritis joints but in different regions (Lechner *et al.* 1997). On the basis of this finding, it is hypothesed that both type of cytokine response could occur concurrently but in different regions of MVV-infected lung tissue. However, studies monitoring changes in levels of IL-10, IL-4 and IFN-γ during MVV infection are required before a definite role of a shift from Th1 to Th2 in the immunopathology of MVV infection can be determined. Whether or not a Th1/Th2 switch occurs, cytokines are certainly critical in modulating the type and the effectiveness of the immune response against lentiviral infection.

1.3.3: **Cytokines induced during lentiviral infection**

1.3.3.1: **Cytokine induction by small ruminant lentiviruses**

Monocytes/macrophages are the major host for the small ruminant lentiviruses MVV and CAEV (Bordie *et al.* 1995). Furthermore, monocytes/macrophages play an important role in immune regulation, both by acting as antigen-presenting cells and by producing cytokines and lipid mediators with potent biological activities. Thus MVV infected monocyte/macrophages play an important role in the pathogenesis of MVV infection. The precise mechanisms are largely unknown, but dysregulation of expression of cytokines by infected monocyte/macrophages has been postulated as a possible mechanism of maintaining or exacerbating inflammation (Lechner *et al.* 1996, Woodall *et al.* 1997). Only few studies have investigated cytokine induction due to MVV infection of host cells. Early studies reported that cytokines, present in complicated lentiviral pneumonia in sheep (Ellis *et al.* 1991), might modulate MVV replication in AMs (Ellis *et al.* 1994). A recent study extended these observations, showing that IFN-γ, IL-1β, IL-4, IL-10, IL-2 receptor mRNA was upregulated in lung tissue of MVV-infected sheep with lung lesions (Woodall *et al.* 1997). In particular, hyperelevation of GM-CSF mRNA was found in lung tissue of MVV infected sheep with lung lesions (Woodall *et al.* 1997). TNF-α and TGF-β mRNA levels were similar to the lung tissue without lung lesions. By contrast, FIV infected

PBMCs have shown an increased expression of TNF-α (Kraus *et al.* 1996) and CAEV-infected macrophages has shown a decreased expression of TGF-β (Lechner *et al.* 1997). AMs bearing MVV capsid protein have been found at the periphery of lung lesions (Brodie *et al.* 1995). These observations suggest that MVV infection and replication may directly result in a dysregulated pattern of expression of cytokines in lung tissues of MVV-infected sheep. However, which type of cell is responsible for this dysregulation of expression of cytokines by MVV remains elusive. Currently, studies of the interaction of MVV with AMs showed that IL-8 gene expression is upregulated as a result of MVV *in vitro* and *in vivo* infection (Legastelois *et al.* 1996). Apart from MVV, two other lentiviruses have also been shown to increase IL-8 expression in infected cells *in vitro*, which suggests that this increase may be a common feature in lentivirus infection. CAEV infection results in increased expression of IL-8 in macrophages (Lechner *et al.* 1997) and HIV also increases the secretion of IL-8 in blood-derived macrophages (MDMs) (Lipschik *et al.* 1993). Lentiviruses, however, are not the only viruses capable of augmenting the expression of IL-8. For example, the retrovirus human T cell leukaemia virus type 1 (HTLV-1) increases IL-8 expression by a NF-κB-dependent mechanism (Baba *et al.* 1996). It is unclear whether MVV and CAEV induce the expression of IL-8 by a similar way. Unlike HIV, MVV and CAEV contain neither TAR-like structures nor NF-κB binding sites in the LTR (Gdovin *et al.* 1992). Therefore, HIV and MVV and CAEV may have different effects on expression of cytokines. The fact that the levels of NF-κB and AP-1 activities was not corrected to the levels of IL-8 expression in CAEV-infected macrophages (Lechner *et al.* 1997), suggests that it is most likely that these cellular factors are not involved in the pathway regulating the increased expression of IL-8 mRNA in macrophages.

1.3.3.2: **Insight from HIV**

1.3.3.2a: **HIV infection modulates the expression of cytokines**

The modulation of cytokine expression in HIV-infected monocytes/macrophages has been studied in much greater detail than other lentiviruses. A number of cytokines have been reported to be up- or down-regulated in monocyte/macrophages from

HIV-infected individuals and after *in vitro* infection (Wright *et al.* 1988, Roux-lombard *et al.* 1989, Kekow *et al.* 1990, Vom *et al.* 1990, Peters *et al.* 1991, Wahl *et al.* 1991, Agostini *et al.* 1992, Trentin *et al.* 1992, Twigg *et al.* 1992, Clerici *et al.* 1993a, 1993b, Fan *et al.* 1993, Lipschik *et al.* 1993, Denis *et al.* 1994a, 1994b, Chehimi *et al.* 1994, Borghi *et al.* 1995). Increased levels of TNF-α, IL-1, IL-6, TGF-β and IFN-α and IFN-β have been found in the sera of HIV infected patients (Agostini *et al.* 1992), However, *in vitro*, no significant difference was observed in spontaneous and LPS-induced IL-1β, IL-6 and TNF-α secretion in PBMCs from HIV-infected individuals and those isolated from uninfected controls (Peters *et al.* 1991,Vom *et al.* 1990). A rise in the levels of IL-1β, IL-6 and TNF-α mRNA has also been observed in LPS-stimulated alveolar macrophages isolated from HIV infected individuals (Sjerra-Madero *et al.* 1994). In another set of reports, monocytes/macrophages secrete IL-6 and TNF-α in response to HIV infection (Merrill *et al.* 1989, Breen *et al.* 1990, Biyx *et al.* 1990, Clouse *et al.* 1991, Esser *et al.* 1991, Mabonndzo *et al.* 1991, Ameglio *et al.* 1994, Berman *et al.*1994b, Sandborg *et al.* 1994, Than *et al.* 1994). High levels of expression of these cytokines as well as IFN-γ and IL-10 are particularly evident in lymphoid tissue, a major site of HIV replication throughout the course of disease (Agostini *et al.* 1992, Emilie *et al.* 1994, Chèret *et al.* 1996, Graziosi *et al.* 1996, Khatissan *et al.* 1996a). In some studies, HIV did not appear to stimulate the secretion of IL-6 and TNF-α by monocytes/macrophages (D'Addario *et al.* 1990, Molina *et al.* 1990, Gan *et al.* 1991, Meltzer *et al.* 1991, Sperber *et al.* 1993, Foli *et al.* 1997). In addition, when macrophages differentiated from *in vitro* HIV-infected blood adherent cells are stimulated with LPS, increased levels of IL-8 gene expression are observed compared to that in control (Lipschik *et al.* 1993). These observations show that HIV infection modulates the expression of cytokines in its host.

A recent study demonstrated that no changes in TNF-α and IL-10 mRNA level were observed in PBMCs whereas enhanced levels of TNF-α and IL-10 mRNA could be detected in lymph node mononuclear cell (LNMCs) and mononuclear cells from brochoalveolar lavages (BALMCs) (Chéret *et al.* 1996). Moreover, expression of IL-

2 and IL-4 was generally detected in PBMCs, but rarely found in lymph nodes in a minority of HIV-infected individuals regardless of the stage of disease (Graziosi *et al.*1994a). These results suggest that the levels of cytokine in sera or other body fluid may not reflect cytokine expression in tissues. Thus, a comparative analysis of the expression of cytokines at different tissues is also important for elucidating the pathological events in affected tissues of lentiviral infection.

1.3.3.2b: Viral gene products modulate the expression of cytokines

Study of interaction of HIV genes products including tat, gp120, with molecules on the surface of the target cells showed that the direct infection of monocytes/ macrophages may be not necessary for the expression of some cytokines and that alternative mechanisms may be involved in this process. Inactivated virus or even HIV envelope gp120 at high concentration can induce cytokine production (Clouse *et al.* 1991, Mabondao *et al.* 1991, Oyaizu *et al.* 1991, Riechmann *et al.* 1991, Ameglio *et al.* 1994). Normal PBMCs produced IL-1, IL-6 and TNF-α on exposure to HIV gp120 (Merrill *et al.* 1989, Wahl *et al.* 1989b, Molina *et al.* 1990, Gessani *et al.* 1994) and inactivated HIV (Nakajima *et al.* 1989). Expression of IFN-γ can be induced by inactivated virus and thus is independent of virus replication (Linnavuori *et al.* 1987, Capobianchi *et al.* 1988, 1992a, Gobl *et al.* 1988, Kikuta *et al.* 1990, Szebeni *et al.* 1991, Gendelman *et al.* 1992, Francis *et al.* 1993). Also, IFN-β can be induced in cultured macrophages by HIV recombinant gp120 (Gessani *et al.* 1994), indicating a role for IFN-β in restriction of virus replication. Yet other studies have suggested that HIV tat protein may have a role in this process (Buonaguro *et al.* 1992, Iwamoto *et al.* 1994, Rautonen *et al.* 1994, Scala *et al.* 1994). Taken together, cytokine genes may be in part regulated at a transcriptional level through signal transduction pathways activated by interaction between HIV protein and its receptor such as CD4. It is unknown, however, whether these effects are of sufficient magnitude to induce elevated levels of cytokines *in vivo*. Recently, Foli *et al.* (1997) have reported that monocytes/macrophages productively infected by HIV do not secrete detectable cytokines IL-6 and TNF-α. However GM-CSF, a cytokine that enhances virus replication in monocytes/macrophages, can induce relatively long-

term secretion of cytokines IL-6 and TNF-α by monocytes/macrophages, even in absence of HIV. Also HIV induces production of IL-6 and TNF-α in monocytes/ macrophages treated with M-CSF. However, whether the MVV gene products have the similar effect on cytokine expression is still unknown.

It is obvious from these reports that interaction of lentiviruses with host cells invariably not only results in the release of one or more cytokines but also leads to a dysregulated pattern of cytokine expression in response to the exogenous stimulation. Not all cytokine mRNAs are affected similarly, and the actual cytokines produced depend on the nature of virus and host cell involved or the kind of stimulus, which suggests that the effects of lentiviruses on the regulation of cytokines may be well more complex and remains elusive. The fact that infected macrophages respond differently from uninfected cells to exogenous stimulation is believed to be of particular significance in the pathogenesis of lentivirus-infected sites. Priming for a dysregulation of cytokine expression in response to exogenous stimulation as result of infection may well be a factor in the persistence of inflammation which ultimately leads to clinical symptoms. Thus elucidation of the alterations in cytokine expression following lentivirus infection is a crucial step in determining immunopathological mechanisms involved in lentiviral disease progression.

1.3.3.3: **The expression of cytokines and virus burden**

Although lentivirus infection induced the expression of cytokines (Berman *et al.* 1994b, Legastelois *et al.* 1996), little is known whether the expression of some cytokines *in vivo* are quantitatively related to lentivirus infection, in particular in MVV infection. Study of the relationship between virus burden in AMs and cytokine expression shows that the increased level of TNF-α in HIV-infected AMs and lymphoid tissue did not directly correlate with viral gene expression in these cells or tissues (Sierra-Madero *et al.* 1994, Cavert *et al.* 1997). Similarly in CAEV infection TNF-α expression did not associate with the degree of viral replication (Lechner *et al.* 1996). The lack of relationship between the degree of viral replication and TNF

mRNA expression, indicates that *in vivo* TNF-α may not be critical for inducing viral replication in its hosts. This suggestion is further supported indirectly by the evidence that the virus in the circulation is mostly produced by continuing cycles of infection (Ho *et al.* 1995, Wei *et al.* 1995, Perelson *et al.* 1996) rather than by induction of gene expression in latently infected cells (Cavert *et al.* 1997). However, it does not exclude that TNF-α and other cytokines such as IL-2 and IL-6, are required in earlier stages of infection to activate cells to a permissive state for replication or to upregulate expression of early viral RNA such as tat and Rev RNA. By contrast in FIV infection, production of TNF-α has been shown to be related to the production of FIV p26 in experimentally infected cats (Kraus *et al.* 1997).

In the case of SIV, expression of cytokines TNF-α/β and IFN-γ was correlated with virus replication in experimentally infected animals (Khatissian *et al.* 1994, 1996a). In particular, production of IFN-α was found at higher levels in animals with a high SIV burden (Khatissian *et al.* 1996b). IFN-α is virally induced. The anti-viral activity mediated by IFN-α may, in turn, contribute to the down-regulation of viral load seen after the acute stage of SIV infection. However, productive SIV infection persists in spite of the IFN-α response (Khatissian *et al.* 1996b). The reasons for this are not clear but could be due to the development of virus resistant to IFN-α during the course of infection. HIV isolates from HIV infected individuals with AIDS were found to have various degrees of resistance to IFN-α. This may also occur in MVV *in vivo* infection. It has been shown that IFN present in culture fluids from MVV infected animals with lung lesions has only moderate inhibitory effects on MVV replication *in vitro* (Lairmore *et al.*, 1988a). The role of such virus resistance to IFN-α in immunopathology of the MVV infection remains unclear.

1.3.3.4: **Pathological role of virus-induced cytokines**

Lentivirus infection invariably results in the release of one or more cytokines (Legastelois *et al.* 1996, Poli *et al.* 1993, 1996, Woodall *et al.* 1997). It is tempting, therefore, to hypotheses that lentivirus infection may cause progression of disease via a profound perturbation of the cytokine network. More precisely, increased

cytokine release may have more damaging than favourable effects in the infected microenvironmental sites. The biological significance of increased expression of IL-8 mRNA in MVV (Lega-stelois *et al.* 1996), CAEV (Lechner *et al.* 1997) as well as HIV-infected (Lipschik *et al.* 1993) macrophages remains to be determined. IL-8 is a potent chemotactant for neutrophils. Under certain conditions, IL-8 can also function as a chemotactant for T lymphocytes (Matsushima *et al.* 1992). It is tempting, therefore, to hypothesis that increased expression of this cytokine may contribute to accumulation of neutrophil and lymphocytes in the alveolar spaces of MVV-associated interstitial pneumonia (Cadore *et al.* 1994, Cordier *et al.* 1992, Lairmore *et al.* 1986) and HIV-associated interstitial pneumonia (Joshi *et al.* 1985). Hyperevelation of GM-CSF mRNA has been reported in the lung tissue of MVV infected sheep with chronic lymphocytic interstitial pneumonia (Woodall *et al.* 1997). Similarly increased expression of GM-CSF was observed in HIV-infected cells (Agostini *et al.* 1992, Lipschik *et al.* 1993). GM-CSF up-regulates antigen-presenting activity of dendritic cells and recruits these cells to be localised inflamed regions of the lung, thus promoting lymphoid follicle development. GM-CSF also mediates abrogation of suppression of T lymphocytes activity in the lung by AMs, causing T lymphocyte accumulation to occur. Thus it is possible that increased levels of GM-CSF in the lung may account for development of lymphoid follicles in the lung (Woodall *et al.* 1997).

Infection of macrophages with CAEV leads to a decrease in the expression of TGF-β (Lechner *et al.* 1997). TGF-β potentially inhibits the proliferation of T and B lymphocytes and production of cytokines (Lechner *et al.* 1997). Decreased activity of TGF-β in the affected sites may therefore result in enhanced proliferation of T and B lymphocytes. It is unclear whether the reduced expression of TGF-β in CAEV-infected macrophages also leads to decreased activity of TGF-β *in vivo*. In MVV infection, TGF-β mRNA levels in the lung tissues of MVV-infected sheep with lymphocytic interstitial pneumonia were found to be similar to the lung tissues without lung lesions (Woodall *et al.* 1997).

Like HIV infection, MVV infection in sheep also causes CNS disease such as encephalomyelitis (Watt *et al.* 1994). However, events leading to the neurological disorder are still unclear, but infiltration by monocytes and macrophages is a consistent finding in the brain of AIDS pateints (Koening *et al.* 1986). In AIDS demeatia, the predominant cells in the CNS are macrophages and microglia which are infected and produce HIV-1. Persistence of IL-1, TNF-α, TGF-β, GM-CSF, M-CSF and IL-6 expression in these cells was observed. Many of these cytokines are chemotactic for monocytes and cause death of oligodendrocytes and destruction of myelin *in vitro* (Robbins *et al.* 1987, Whal *et al.* 1991). In one study, increased level of IL-1β and IL-6 was observed in the cerebrospinal fluid. Therefore cytokines produced by resident macrophages and microgila cells may contribute to many of clinical and histological effects of lentiviral infection in the CNS. Undoubtedly, these findings support the suggestion of a possible pathogenic role for cytokines in the virus-infected micro-environmental sites.

1.3.4: **Role of cytokines in regulation of lentivirus expression**

It has been speculated that the interaction of the viral genome with cellular factors may play a critical role in induction of lentivirus expression (Stanley *et al.* 1989, 1990, 1996, Ho *et al.* 1992, Poli *et al.* 1992a, 1993, O'Brien *et al.* 1995, Staprans *et al.* 1995). In this scenario, the cytokine network has emerged as an important component of host-mediated control of virus replication (Paul *et al.* 1994). The cytokine produced determines the immune response to viral infection, which in turn regulates virus replication and determines the persistence of virus *in vivo*. Therefore, the role of cytokine and cytokine dysregulation in the pathogenesis of lentiviral infection is under intense investigation. The finding that culture supernatant from PBMCs stimulates HIV expression in chronically infected cells lines (Folks *et al.* 1987) led to the identification of numerous individual cytokines that have either stimulatory or inhibitory effects on lentivirus replication either when manipulated endogenously or when added to acutely or chronically infected cultures (Vincenzi *et al.* 1994).

1.3.4.1: **Stimulatory effects of cytokines on the virus replication**

An enhanced effect of TNF-α on MVV expression in AMs has been shown *in vitro* (Ellis *et al.* 1994). Similar effects of TNF-α were also observed on HIV in T lymphocyes and mononuclear phagocytes (Folks *et al.* 1989, 1987, Clouse *et al.* 1989a, Matsuyanma *et al.* 1989) and SIV in simian AMs (Walsh *et al.* 1992). It is obvious from these reports that if this effect occurs *in vivo*, TNF-α could be an important mediator of disease *in vivo*, in particular in the case of complicating secondary infections (Ellis *et al.* 1994). It is unclear how TNF-α enhances MVV replication in infected AMs. Previously, it has been shown that TNF-α activates SIV expression in rhesus macaques macrophages on a per cell basis (Walsh *et al.* 1992), suggesting that cytokine treatment results in an increase in the amount of lentiviruses produced per infected macrophage. Enhanced effects of TNF on MVV and SIV expression in AMs may occur through the transcriptional activation of lentiviruses as a result of TNF-α induction. It has been shown that TNF-α leads to induction of HIV expression via the activation of the cellular transcription factor NF-κB (Duh *et al.* 1989, Griffin *et al.* 1989, Osborn *et al.* 1989). Activation of NF-κB involves the phosphorylation and degradation of I-κB (Lenardo *et al.* 1994), allowing NF-κB to translocate to the nucleus and bind to promoter region of several cellular genes as well as consensus sequences present in HIV-LTR. TNF-α receptors are engaged by TNF-α and TNF-β via sphingomyliase and ceramide (Parrott *et al.* 1991, Schutze *et al.* 1992) which inactivate I-κB. In this regard, TNF can potentially contribute to the generation of viral quasispecies by rescuing defective viruses otherwise incompetent for replication. Binding of NF-κB to the HIV LTR leads either to initiation or to potentiation of viral transcription. However, unlike HIV and SIV, MVV contains neither TAR-like structures nor NF-κB binding sites in the LTR (Gdovin *et al.* 1992). Therefore cytokines may regulate HIV MVV in different way. Considering the fact that there is no difference in the levels of binding activity of NF-κB between uninfected and CAEV-infected macrophages (Lechner *et al.* 1997), it is suggested that TNF enhances MVV replication in infected AMs by a NF-κB-dependent mechanism. A recent study (Herbein *et al.* 1996) has demonstrated an additional role for TNF-α in terms of inhibitory capacity on entry of HIV into primary

macrophages. This inhibition of HIV entry by TNF-α is independent of CD4 expression level and occurs in tissue culture differentiated macrophages but not in PHA-activated PBMCs. However, it is unclear which entry step (virus-cell binding, fusion or uncoating) is inhibited by TNF-α.

IL-1 is a pleiotropic cytokine consisting of two distinct but related proteins, IL-1α and IL-1β, and is mainly produced by macrophages (Akira *et al.* 1990). IL-1 is involved in regulating the growth and differentiation of lymphocytes and induction of acute inflammation. IL-1 has been shown to stimulate MVV replication in AMs (Ellis *et al.* 1994). Similarly, IL-1 can enhances HIV replication or production *in vitro* (Poli *et al.* 1994, Kinter *et al.* 1995). Although IL-1 is thought to affect viral transcription, it is unclear whether the mechanism of IL-1 enhancement of HIV involves the activation of NF-κB as in TNF stimulation.

Other cytokines have also been shown to have a stimulatory effects on HIV replication *in vitro*, including IL-2 (Clouse *et al.* 1989b, Arai *et al.* 1990, Smithgall *et al.* 1996), IL-6 (Poli *et al.* 1990), and IL-7 (Smithgall *et al.* 1996). But the role of these cytokines in regulating viral replication has not yet been studied for MVV.

1.3.4.2: **Inhibitory effects of cytokines on the virus replication**

The effects of lentivirus-stimulating cytokines are counterbalanced by those that down-regulate virus replication. IFN-α/β, as prototypes of cytokines, are naturally occurring proteins capable of eliciting anti-viral and immunoregulatory activities. They are produced principally in macrophages, B cells (IFN-α) and fibroblasts (IFN-β) in response to viral infection. INF-α and INF-β bind to a common ubiquitously expressed cell surface receptor leading to induction of interferon-stimulated genes, such as PRK and 2', 5'-oligoadenylate synthetase, for cellular anti-viral and immunoregulatory activities (Samuel *et al.* 1993). IFN present in culture fluids of cells derived from animals with lymphocytic interstitial pneumonia has been shown to have moderate inhibitory effects on MVV production *in vitro* (Lairmore *et al.* 1988a). Furthermore, as in the *in vitro* study of with 'leukocyte-derived IFN in

CAEV-infected monocytes (Zink and Narayan 1989), bovine recombinant IFN-α has an substantially inhibitory effect on MVV replication in macrophages (Ellis *et al.* 1994).

Similar observations are reported for HIV. *In vitro*, Both IFN-α and IFN-β exert similar inhibitory effects on HIV replication (Kornbluth *et al.* 1989, Gendelman *et al.* 1990a, 1990b, Shirazi and Pitha 1992, Gessani *et al.* 1994, Vincenzi and Poli 1994). The mechanism of this inhibition in monocyte/macrophages of lentiviral production was dependent on the time of exposure to IFNs (α/β). For example, in infected primary macrophages, when addition of IFNs and infection occur at the same time, IFNs interfere with an early step of HIV replication cycle virus by preventing formation of HIV-1 provirus (Poli *et al.* 1989, Kornbluth *et al.* 1990, Meylan *et al.* 1993), indicating IFNs may influence virus receptor binding, fusion, un-coating, or reverse transcription. In contrast, when addition of IFNs occurs after infection, IFN reduces the relative level of viral RNA without affecting formation of HIV-1 provirus (Gendelman *et al.* 1990a, 1990b). It is obvious that the IFN-mediated inhibition of lentiviral replication occurs at multiple levels, *in vivo*, and IFN may have a broad spectrum of anti-cellular and anti-viral activities (Poli *et al.* 1989, Dubreuil *et al.* 1990, Gendelman *et al.* 1990a, 1990b, Kornbluth *et al.* 1990, Smith *et al.* 1991, Shirazi *et al.* 1992, Meylan *et al.* 1993).

Recently, another lentivirus inhibiting IL-16 was found in humans and monkeys. IL-16 is secreted from CD8+ cells and induces a migratory response in lymphocytes, monocytes and eosinophils expressing CD4 molecules (Laberge *et al.* 1995). Cloned African green monkey IL-16 and human IL-16 share 95 % homology (Baier *et al.* 1995). Either African green monkey IL-16 or human IL-16 has been shown to inhibit HIV replication in CD8+-depleted PBMCs (Baier *et al.* 1995) but high concentrations were required for inhibition. In comparison, the human IL-16 was less potent than the monkey IL-16. These data in part explains why monkeys do not succumb to SIV. Sheep IL-16 has been reported yet. It is unclear whether IL-16 has similar effects on MVV replication.

1.3.4.3: **Bifunctional cytokines**

A level of complexity exists in terms of the regulatory effects of cytokines on lentiviral replication. In some cases, cytokines either induce or inhibit viral replication *in vitro* as a function of the model systems. In regard to HIV infection, IFN-γ and TGF-β reduce or enhance viral replication depending on the infected cell type and the culture condition (Koyanagi *et al.* 1988, Lazdins *et al.* 1991, Poli *et al.* 1991, Biswas *et al.* 1992, Fan *et al.* 1994, Dhawan *et al.* 1995). Opposing effects were also noted in the cases of IL-4 and IL-13 when freshly isolated monocytes or matured macrophages are exposed to HIV (Kazazi *et al.* 1992, Schuitemaker *et al.* 1992, Naif *et al.* 1997). For example IFN-γ treatment prior to infection has a predominantly stimulatory effect on HIV replication, whereas postinfection treatment with IFN-γ results in decreased virus expression (Koyanagi *et al.* 1988). Thus, IFN-γ may act as a negative and positive regulator of viral replication as a function of variables which remain unclear, but appear to be linked to experimental conditions, in particular the time of IFN-γ addition and the type of cells infected, the activational state of the infected macrophages at the time of exposure to IFN. It is also possible that factors co-expressed together with IFN-γ or induced as a consequence of the production of this cytokine may also account for some of these discrepant findings. The bifunction of IFN may in part explain the observation that the level of IFN spontaneously secreted by pulmonary leukocytes was consistent with increased MVV replication in tissues and more severe lymphocytic interstitial pneumonia in lambs experimentally infected with MVV (Lairmore et al 1988a).

TGF-β is secreted primarily by platelets and macrophages (Massague *et al.* 1990). It has a broad range of biological activities which vary depending on cell type, state of differentiation, and the presence of other cytokines (Massague *et al.* 1990). Similar to the effects of IFN-γ, TGF-β has been shown to regulate bifunctionally viral replication *in vitro* (Lazdins *et al.* 1991, Peterson *et al.* 1991, Poli *et al.* 1991). However, the effects of TGF-β *in vivo* remain unclear. It is possible that the effects of TGF-β in HIV replication *in vivo* vary with the phase of the infection and the

context of other cytokines that modulate virus replication. No study of the effect TGF-β on MVV has been made.

GM-CSF, a member of GSF family, is a key regulator of monocyte-macrophages. It is required for the survival, proliferation, and differentiation of monocyte-macrophages and for the function of the mature progeny (Metecalf *et al.* 1992, Crowe and Lopez 1997). The ability of monocytes-macrophages to harbour MVV and to allow its replication suggests that cytokines which affect the production of monocytes, their differentiation and functional status, may modulate MVV replication *in vivo*. In fact, differentiation and activation of these cells is associated with lentiviral infection and replication, thus suggesting that GM-CSF may affect viral replication at multiple stage of monocyte-macrophages differentiation (Poli *et al.* 1992b). However, little is known about effects of GM-CSF on MVV infection of monocyte/macrophages.

The effects of this cytokine on HIV replication have been studied in more detail. The early studies of the effects of GM-CSF on HIV replication have shown that GM-CSF has a significant increased effect on the expression of HIV-1 (Folks *et al.* 1987, Koyanagi *et al.* 1988, Perno *et al.* 1989, Shuitemaker *et al.* 1990, 1992, Perno *et al.* 1992). The mechanism of enhancement of HIV replication by GM-CSF remains unclear. The possible ways that GM-CSF may augment expression of HIV in monocyte-macrophages is through the stimulation of host-cell transcriptional factors which could bind to the HIV LTR, resulting in increase in HIV replication (Nabel *et al.* 1987). The region responsible for GM-CSF activation in these cells is located slightly upstream of, and partially overlaps, the NF-κB binding region (Zack *et al.* 1990). In contrast to its stimulatory effects on HIV replication, GM-CSF also has a negative effect on HIV replication (Hammer *et al.* 1986). HIV replication in monocyte-derived macrophages stimulated with GM-CSF is suppressed in contrast to active production in macrophage induced by M-CSF (Matsuda *et al.* 1995), although the expression of CD4 on the surface of M-CSF and GM-CSF stimulated cells is similar, In GM-CSF-stimulated macrophages the level of proviral DNA is

much less than that in M-CSF treated macrophages, suggesting that the suppression of HIV replication in macrophages induced by GM-CSF may be largely due to disturbance at certain steps of replication after synthesis of proviral DNA. The reason for this discrepancy could be differences in the cells, the stage of maturation of monocytes both at the time of cytokine exposure and at the time of infection, virus strains, and culture conditions (Folks *et al.* 1987, Hammer *et al.* 1990, Matsuda *et al.* 1995, Feng *et al.* 1996). Considering the facts that GM-CSF has both suppressive or stimulatory effects on HIV replication in macrophages *in vitro* and that there is the increased expression of GM-CSF in the lung tissue of MVV-infected sheep (Woodall *et al.* 1997), it is possible that GM-CSF is involved in regulating MVV replication *in vivo*. It would be important to know whether GM-CSF has a similar effect on MVV replication in macrophages as has been demonstrated in HIV. Thus further study is required to clarify this point.

IL-10 is a pleoiotropic cytokine which is associated with the negative regulation of IL-12 and IFNγ and to development of Th2 type immune response (More *et al.* 1993). IL-10 has multiple effects on macrophages including inhibition of cytotoxicity, oxidative burst, nitric oxide and cytokine production (IL-6, TNF-α and IL-1β) and induction of IL-1ra and FcγR1 (Moore *et al.* 1993). That implicates IL-10 as an important factor in the regulation of cell-mediated immune responses to viral infection (Clerici *et al.* 1994). It has been demonstrated that IL-10 has dichotomous effects on HIV replication (Montanier *et al.* 1994, Weissman *et al.* 1994, 1995). IL-10 can markedly inhibit HIV replication in monocyte/macrophages at concentration that block secretion of endogenous cytokines such as IL-6 and TNF-α which can up-regulate HIV-1 expression in these cells (Montanier *et al.* 1994, Weissman *et al.* 1994). The mechanism of suppression of HIV replication by IL-10 remains unclear although it is thought to decrease HIV-1 reverse transcription upon macrophage infection and subsequently mediate viral latency *in vitro* (Montanier *et al.* 1994). The inhibition of HIV replication in monocytes by IL-10 is associated with inhibition of cell differentiation (Chang *et al.* 1996). In contrast, IL-10 at concentrations that do not completely inhibit release of these endogenous cytokines

actually enhances HIV replication in infected monocyte/macrophages (Weissman *et al.* 1995). On the basis of these observations, it is possible that there is a synergistic effect between the exogenously added IL-10 and endogenously secreted cytokines (Weissman *et al.* 1995, Finnegan *et al.* 1996).

The Th2 cytokines IL-4 (Novak *et al.* 1990, Schuitemaker *et al.* 1992, Naif *et al.* 1997) and IL-13 (Naif *et al.* 1997), released by activated Th2 lymphocytes (De Waal Malefyt *et al.* 1993), also have dichotomous effects on HIV replication. Interestingly, although IL-4 and TNF-α can up-regulate HIV replication independently, combination of both cytokines resulted in a partial inhibition of the maximal effect of TNF-α (Naif *et al.* 1997). It is possible that IL-4 inhibits TNF-α enhancement of cytokine gene transcription by macrophages (Essner *et al.* 1988, Yanagawa *et al.* 1991).

1.3.4.4: **Interaction between cytokines in lentivirus infection**

The effects of a particular cytokine are often greatly influenced by the activity of other cytokines present in microenviroment. In this regard, certain cytokines have been shown to act in a synergistic or in an antagonistic manner with other cytokines in regulating viral replication. It has been reported that IL-6 co-operates with TNF-α (Poli *et al.* 1990) or IL-1 (Poli *et al.* 1994) to induce an increase in HIV replication greater than either cytokine alone. In addition, synergy for up-regulation of viral expression can also occur between cytokines and cAMP or glucocorticoids. For example, TNF-α co-operates with cAMP (Chowdhury *et al.* 1993) or glucocorticoids (Bressler *et al.* 1993) to stimulate HIV expression in latently infected cells of monocyte-macrophage lineage. Finally, cytokines are pleiotropic and the overall effects may reflect the balance between lentivirus-inducing and inhibiting-activities. From these observations, it is obvious that interaction of cytokines with lentivirus infection is more complex. Thus the stimulatory and/or inhibitory effects of many cytokines on the viral replication are observed *in vitro*, but it does not address the possible role of endogenous cytokines in controlling the viral replication *in vivo* and a direct correlation with cytokine effects *in vivo* remains difficult to establish. The

important point about these observations is that a group of cytokines is capable of either regulating the induction of lentiviral expression from the state of latent or chronic infection to active viral expression or down-regulating lentiviral replication. In comparison with HIV and SIV, little information is available regarding the induction of MVV expression by cytokines.

1.4: **Aims**

Macrophages are the host cells for replication of MVV (Brodie *et al.* 1992, 1995). However, the level of viral replication and load in AM and its correlation with pulmonary lesion is unknown. Also it is not yet clear whether cytokine expression is dysregulated in macrophages infected with MVV. In addition, the effects of exogenous cytokine on MVV replication in macrophages have not been documented.The aims of this study were to determine 1. the level of MVV DNA in AM compared with blood monocytes in natural infection and assess the correlation of viral burden and replicative status to the lung lesions related to MVV infection; 2. the differential expression of cytokine genes in macrophages infected with MVV *in vivo* and *in vitro*; 3. the effect of GM-CSF, TGF-β and IFN-γ on MVV replication in macrophage *in vitro*. Prior to this a QC-PCR had to be developed for quantitative molecular analysis of viral activity *in vivo*.

CHAPTER 2

MATERIALS AND METHODS

2.1: **Materials**

All chemicals were supplied by SIGMA Chemicals Co., England unless otherwise stated.

2.2: **Methods**

2.2.1: **Cell culture**

2.2.1.1: **Cells for propagation of MVV**

2.2.1.1a: **Sheep epidermal fibroblast cells**

Sheep epidermal fibroblast cultures were grown in Dulbeco's modified Eagle's medium (DMEM) (Gibco) supplemented with 2mM L-glutamine, 100u/ml penicillin and 200µg mg/ml streptomycin and 10% (v/v) foetal calf serum (FCS). When the monolayer was approximately 90% confluent, medium was removed and cells were washed with Versene and then treated with 0.05% (w/v) trypsin and 0.02% (w/v) EDTA in phosphate buffered saline (PBS) (0.8% NaCl, 0.02 % KCl, 27mM Na_2PO_4, 1.5mM KH_2PO_4, PH7.2) until cells were no longer adherent. 10 ml of fresh medium was added and cells split 1:3. Cells were routinely grown at 37°C with 5% CO_2 in tissue culture flasks. Cells were stored in liquid nitrogen at a concentration of 1 x 10^6/ml in 90% (v/v) FCS/10% (v/v) DMSO. Cells were resuscitated by thawing quickly at 37°C and washed twice with 10% (v/v) /DMEM to remove DMSO.

2.2.1.1b: **Preparation of virus stocks**

All experiments used the EV1 isolate of maedi-visna virus (Sargan *et al,* 1991). The virus was passaged in the fibroblast culture maintained in DMEM/8% (v/v) FCS. Briefly, when the monolayer was approximately 90% confluent, medium was removed and virus was added. After incubation at 37°C for 1 hour, fresh DMEM/2% (v/v) FCS was added. Virus containing supernatant was harvested when the culture

displayed extensive cytopathic effect and clarified by centrifugation at 8 000rpm for 10 minutes at 4°C. The supernatants were aliquoted and stored at -70°C until used.

2.2.1.1c: **Titration of virus**

96 Well flat-bottomed tissue plates were seeded with 1x 10^4 cells per well in 100µl of DMEM containing 8% (v/v) FCS and incubated at 37°C overnight. 10-fold virus dilution was made in DMEM containing 2% FCS (v/v). Medium was removed from the inside 60 wells of the plate, and virus dilutions were added in columns (e.g. six wells for each dilution), incubated at 37°C for 1-2 hours. Medium was used as mock infection control. 50 µl of DMEM containing 2% FCS (v/v) was added into each well. The cells were fed with another 100µl of DMEM containing 2% FCS (v/v) 4 days later. After incubation for another 3 days. cells were stained as follows.

After removal of medium, cells were fixed with ice-cold acetone/methanol (1:1), 100µl per well for 5 minutes at room temperature. After removal of acetone/ methanol and air dried slightly, cells were then treated with 1% (w/v) potassium dichromate for 30 minutes, 50 µl per well and re-fixed with acetone/methanol as before. Cells were stained with Giemsa stain freshly diluted in water (1:1) for 5 minutes, 100 µl per well. Cells were then washed in excess of water and destained in tap water until the cell nucleus was dark blue and the cytoplasm light blue. The plate was air dried and examined for presence of syncytia. The 50% tissue culture infectious dose ($TCID_{50}$/ml) was calculated by Reed and Meunch's method.

2.2.1.2: **Isolation and culture of macrophages**

2.2.1.2a: **Animals**

Samples including serum, blood, bronchoalveolar lavage fluids were collected from a heavily infected flock of sheep belonging to the Pathology laboratory under controlled conditions. All the sheep were Texel breed apart from Sh106 which was a Bleu de Maine. They were aged between 3 and 10 years of age at the time when the samples were collected. Previous examination of the source flock revealed no evidence of interrecurrent respiratory disease that was unassociated with MVV

infection (Watt *et al.* 1992). Sheep from flocks not infected with MVV were purchased locally. Uninfected and infected animals were separately housed but otherwise managed under similar conditions. Animals were divided into two groups according to histological lesions in lung: Group A consisted of sheep with lung lesions and Group B consisted of sheep without lung lesions.

2.2.1.2b: **Isolation and culture of alveolar macrophages**

Animals were euthanatized by intravenous injection of barbiturate and were exsanguinated. The lungs and trachea were removed intact. The lung was then flushed via the trachea with 200 ml of sterile PBS or HBSS in four aliquots of 50 ml each. A gentle aspiration of the bronchoalveolar lavage fluid (BALF) is carried out after each infusion and the four recoveries are pooled together in precooled plastic containers. BALF was filtered through a loose cotton gauze. Cells were recovered by centrifugation (800g, 4°C, 10-20min depending on the volume of BALF). Cell pellets are washed twice in RPMI-1640 supplemented with 2 % (v/v) FCS, 2mM L-glutamine, 100u/ml penicillin and 200µg/ml streptomycin. Cells counts and viability were performed by using trypan blue exclusion and cells were adjusted to 4 x 10^6 cells/ml in PRMI/10 % (v/v) FCS. 5 ml of cells are seeded in 25 cm^2 tissue culture flasks at 37°C with 5 % CO_2 in air. After 1or 2 hr, non-adherent cells and adherent cells are harvested and the cells are refed with fresh medium. Adherent cells recovered were used immediately for either cytospin immunostaining or for RNA or DNA preparation. For culture, one ml of adherent cells at a concentration of 10^6/ml was dispensed into the well of 24-well plate prior to treatment. To harvest adherent cells (MDMs or AMs), cultures were washed with PBS once and 2.5 ml of 5mM EDTA in PBS is added to the flasks, after 2-5min incubation, the flasks are knocked to dislodge the cells and a rubber policemen used to scrape off any remaining adherent cells.

2.2.1.2c: **Isolation and culture of macrophages from sheep blood**

Blood samples were collected in an EDTA K3 containing vacutainer (Becton Dicknson) and diluted by addition of an equal volume of sterile physiological saline

(0.9% w/v NaCl). The diluted blood was loaded onto the surface of Lymphoprep (Nycomed, Norway) and centrifuged at 2000rpm at room temperature for 20-40 min, and then peripheral blood mononuclear lymphocytes (PBMC) were collected and washed in sterile PBS three times (by centrifugation at 1200 rpm, 10min). The cell pellets were re-suspended in RPMI medium supplemented with 10% FCS, 2mM L-glutamine, 2.5mM HEPES, 100u/ml penicillin and 200µg/ml streptomycin at a concentration of 2-4 x 10^6 cells/ml. 5 ml of the cell suspension was added to 25cm^2 tissue culture flasks and incubated at 37°C for 2 hrs before non-adherent cells were removed, and then the medium was replaced in adherent flasks.

2.2.2: **Preparation and purification of proteins**

2.2.2.1: **Preparation of recombinant p25**

2.2.2.1a**: Purification of Ty-p25 from yeast**

Ty-p25 was purified from yeast by a modification of the method of Reyburn *et al.*(1992). Briefly, yeast cells were harvested and washed once in ice-cold sterile distilled water (SDW). All subsequent procedures were carried out at 4°C using pre-chilled buffers. Cell pellets were re-suspended and washed twice in 20 ml of TNE buffer (10mM Tris HCl, pH 7.4, 2mM EDTA, 140mM NaCl), and then cell pellets were re-suspended in 4 ml of TEN, 5 ml acid-washed and baked glass breads were added and the yeast cells were disrupted by three rounds of vortexing with glass breads (during which they were vortexed for 10x 30sec., bursts with 30sec., rest on ice in-between). After each round of vortexing the suspension was centrifuged (5000rpm, 4°C, 10min). The supernatant was collected and kept on ice, the pellets were re-suspended and washed twice in TEN, More than 90% breakage of yeast cells was achieved. All supernatants were pooled and clarified by centrifugation (10500 rpm, Beckman SW40, 4°C, 20min) and then concentrated onto a 2 ml 60 % sucrose cushion (30000 rpm, Beckman SW40, 4°C, 20min). Material on the cushion were collected and dialysed overnight against a 1L of TEN at 4°C. Ty-p25 fusion proteins were purified from the dialysate by centrifugation (24000 rpm, Beckman SW40, 4°C, 3 hrs) through sucrose gradient (15%, 25%, 35%, 45% sucrose solution). After centrifugation, the gradient was fractionated into 2 ml aliquots and fractions

containing Ty-p25 were identified by analysis on SDS-PAGE. The fractions containing Ty-p25 were pooled and dialysed against several changes of Factor Xa buffer (100mM Tris HCl, pH 7.4, 10mM $CaCl_2$) over 36 hours at 4°C

2.2.2.1b: **Purification of p25**

The dialysate was made to 0.05% CHAPS (3-[(-cholamidopropyl) dimethyl-ammonio]-1-propane sulphonate) and 0.05% DOC (deoxycholic acid) and incubated with restriction protease Factor Xa (BCL) at a ratio of 50:4 at 22°C overnight. An aliquot of this digest was analysed by SDS-PAGE to check that the digestion was complete. The suspension was then centrifuged (31000 rpm, 4°C, 1 hr) to pellet the Ty-VLPs. The supernatant containing p25 was dialysed against two PBS changes at 4°C and stored in aliquots at -70°C. Purity of antigen was assessed by SDS-PAGE.

2.2.2.2: **Purification of monoclonal antibody against recombinant p25**

McAb IgG from cell supernatant was purified by using a protein G Sepharose 4 fast flow column (Pharmacia). A 5 ml protein G Sepharose 4 fast flow column was equilibrated with five bed volumes of 0.02 M sodium phosphate (pH 7.0) at a flow rate of 60ml/h. The sample was loaded onto the column and the unbound protein was washed out with the same buffer as above until no protein was detected in the eluant by UV at 280nm. The bound protein was eluted with 0.1M glycine-HCl (pH 2.7) and 0.5 ml fractions were collected in tubes containing a sufficient amount of 1.0M Tris-HCl (pH9.0) (60µl-100µl per ml fraction). The fractions containing protein were pooled and dialysed into PBS overnight at 4°C. The protein concentration was measured by UV at 280nm. The column was washed extensively with PBS between application of each sample and finally was washed and stored in PBS/20% ethanol. The protein concentration was measured by UV at 280nm and stored in -20°C

2.2.3: **Immunoassays**

2.2.3.1: **Quantitation of MVV Gag p25**

Supernatants from cell culture were assessed for MVV Gag p25 by liquid-phrase blocking enzyme-linked immunosorbent assay (ELISA) as described in appendix.

Briefly, ELISA plates were coated with purified recombinant p25. Supernatants were incubated with a pretitrated, known concentration of recombinant p25, and then added into the coated plates. The plates were developed with anti-mouse IgG-alkaline phosphatase. Under these conditions, the assay could detect as little as 500pg to 1ng of MVV p25 per ml. Spectrophotometric readings were converted into inhibition (%) which were calculated as follows. Inhibition (%) = (OD value of non-blocking control – OD value of sample)/OD value of non-blocking control. Inhibition was then converted into ng/ml p25 antigen from a standard curve derived from serial titration of known quantities of purified recombinant MVV p25.

2.2.3.2: **Immunochemistry staining**

The single staining technique was used to detect expression of viral protein or cell surface marker and performed using commercial streptavidin-biotin kit (Vector Laboratories). Reagents were made up according to the manufacturer's instruction. Incubation was performed at room temperature and washes were carried out with PBS for 10 minutes each. Cytocentrifuge preparation were air-dried and re-hydrated by incubation for 10 minutes in PBS, and then incubated in a solution of 3% H_2O_2 in methanol, to inactivate endogenous peroxidase activity. Smears were rinsed and incubated with normal rabbit serum for 30 minutes to block nonspecific interaction of antibodies with samples and then incubated with primary McAb antibody overnight at 4°C. McAb was diluted in PBS containing 1 % normal serum. After overnight incubation, smears were re-equilibrated to room temperature and washed three times with PBS. Biotinylated rabbit anti-mouse IgG, diluted in 1% normal mouse serum /PBS, was then added to cytocentrifuge preparations and incubated for 30 minutes and washed another three times with PBS. The slides were incubated with horseradish peroxidase-conjugated streptavidin for 30 minutes and washed again. HRP substrate solution diaminobenzidine (DAB) chromogen, diluted in H_2O according the manufacturer's protocol, was added to the slides and incubated for 5 minutes. The reaction was stopped by washing with tap water. The slides were slightly counterstained with Mayer's hematoxylin for 5 minutes and dehydrated through graded concentrations of ethanol and xylene and then mounted.

Cytocentrifuge smears from seronegative animals and from seropositive animals previously tested were used as negative and positive control, respectively, to test the specificity of the staining. Positive cells were counted in cytocentrifuge preparations in 20 randomly selected high-power (400 x) and the positive cell mean was obtained.

2.2.3.3: **Flow cytometry analysis of cells for expression of surface molecules**

The code name, specificity, dilution and source of the panel of antibodies used in this study are summarised in Table 6.1. All of them are mouse McAbs. The cells were incubated with McAb on ice for 15 minutes in phosphate-buffered saline (PBS), which was supplemented with 10 % BSA to block non-specific binding of McAb via FcR. The cells were washed twice in PBS and incubated with FITC-conjugated anti-mouse IgG (diluted in PBS/10%BSA/0.1%NaN_3) on ice for 15 minutes. The cells were washed as before and re-suspended in PBS/10% BSA/0.1%NaN_3 Fluorescence intensity was analysed on a FACScan Plus (Becton-Dickinson). Scatter characteristics was used to gate the monocytes and 5000 cells were analysed. Per sample data was expressed as a percentage of positive cells or the mean fluorescence intensity after subtraction of the mean fluorescence intensity of the isotype control (arbitrary units).

2.2.4: **Molecular biology**

2.2.4.1: **Preparation, purification and analysis of nucleic acid**

2.2.4.1a: **Extraction of DNA**

AM and PBMC were obtained from MVV infected and control sheep. The DNA was extracted with a Qiagen kit (Qiagen), according to the manufacturer manual. DNA quality and amount were determined spectrophotometrically and by agarose gel electrophoresis. The DNA from each sample was co-amplified in the presence of competitive template DNA to measure quantitatively MVV DNA present in each sample described subsequently.

2.2.4.1b: **Isolation of RNA**

Total RNA extraction was done using RNeasy Total Kit (Qiagen) according to

manufacture's instruction. Briefly, cell pellets were mixed with 0.6 or 1.2 ml of lysis buffer RLT (containing guanidinium isothiocyanate, 2-mercaptoethanol). The mixture was homogenized using a syringe and sequentially added onto the surface of RN easy spin column and centrifuged at 10000rpm for 15 sec. Next, the column was washed once with wash buffer RW1 and then twice with another wash buffer RPE. The total RNA was eluted with 20-40 µl of diethylpyrocarbonate (DEPC) treated water by centrifugation (10000rpm, 1min).

2.2.4.1c: **Preparation of plasmid DNA**

Plasmid DNA was prepared by using the Qiagen purification method (Qiagen) following the manufacturer's instruction. Plasmid mini-preps were prepared by using Qiagen Plasmid Spin kits (Qiagen), starting with 1 ml of bacterial culture grown for a single colony overnight as described in appendix.

2.2.4.1d: **DNA gel electrophoresis**

DNA samples were analyzed on a 2% (w/v)/TAE gel containing 1 µg/ml of ethidium bromide. DNA samples were mixed with loading buffer in a ratio of 1:5 loading buffer to sample and then loaded onto the gel, followed by running at a constant voltage of 80 - 100 mV for 1 to 3 hours. DNA in the gels was visualized on a UV transilluminator. 1Kb DNA ladder (Gibco) was run alongside the samples.

2.2.4.1e: **Recovery of DNA from agarose**

DNA was run on a 1 % (w/v) low melting point agarose made up in TAE buffer. DNA in the gel was visualized by UV illiumination and the predicted size of DNA fragment cut out of the gel. DNA was extracted and purified by using the Geneclean II kit (BIO 101 Inc.) as described in appendix.

2.2.4.1f: **Measurement of DNA and RNA concentration**

Concentration and purity of DNA and RNA was determined by measuring the absorbance at 260nm and 280nm in a spectrophotometer. An absorbance of 1 unit at 260nm is equivalent to 50 µg of double stranded DNA per ml or 40 µg of single

stranded DNA or RNA per ml. Purity of the DNA or RNA was determined by calculating the ratio between the absorbance values at 260nm and 280nm, where pure DNA or RNA has a ratio of 1.7-2.0. For small amount of DNA, estimates were taken by visualising the intensity of the fluorescence under UV illumination compared to DNA markers of the known concentration.

2.2.4.2: **PCR amplification**

2.2.4.2a: **Reverse transcription of RNA**

In the lid of a 1.5 ml reaction tube, 13.5µl of RNA containing sample was added to the premix, consisting of 4 µl of 5xRT buffer (250mM Tris HCl, pH 8.8, 375mM KCl), 2µl of 20mM dNTP mix, 1 µl of random hexamers (1u/µl, Pharmacia). The tube was closed and was then incubated for 3min at 80°C. Subsequently, the tube was placed on ice and spun down, and 1µl (200 units) of Superscript II Reverse Transcriptase (Life Technologies) was added. This reaction was incubated for 1 hr at 45°C and then heated to 95°C for 5min. Subsequently, the reaction mixture was placed on ice and then spun down and, finally, diluted by addition of 20 µl of RNase free water.

2.2.4.2b: **Primers**

The oligonucleotide primer sequences were chosen from the highly conserved regions (POL, LTR, GAG) of MVV genome (Table 2.1). Various considerations were taken when designing primers. Primers were made between 20-25 long and contained no palindromic sequences to prevent formation of hairpin structures. The primers were synthesized by Genosys (Genosys Biotechnologies (Europe) Ltd, UK).

2.2.4.2c: **PCR amplification**

A 3 µl of cDNA or DNA sample was added to 46 µl of the PCR mixture. The PCR mixture consisted of 10mM Tris HCl (pH8.3), 50mM KCl, 2.5mM $MgCl_2$, 200µm each 2'-deoxynucleoside 5'-triphosphate, 0.5pM sense primer and 0.5pM anti-sense

Table 2.1 Oligonucleotide primers used

Primers	Orientation	Sequences(5' to 3')	Product length	Position
POL	Sense	ATAGTAAATGGCATCAAGATGC	217	4231
	Anti-sense	TCCCGAATTTGTTTCTACCC		4448
	Probe	CATTGGCAAGTGGATT		
LTR	Sense	GACTGTCAGGGCAGAGAACAG	156/116	8912
	Anti-sense	GCATCAGCTCATACATGACT		9068
	Probe	TGCCCATGATTGAGAATGACTATGTA		
GAG	Sense	TAGAGACATGGCGAAGCAAGGCT	461	496
	Anti-sense	TCCTGCTTGCAAATTTACAATAGG		960
	Probe	AATGCCCATAGACAGTTCCCTCTG		

primer. The reaction mixture was overlaid with 30 µl of light mineral oil and placed in a thermal cycler (Perkin-Elmer Cetus). The reaction mixture was initially denatured at 95°C for 5 min before 1 µl (1unit) of Taq DNA polymerase (Gibco) was added, followed by 35 cycles of reaction parameters: 50s of denaturation at 95°C, 1min of primer annealing at 55°C, and 2 min of primer extension at 72°C. The final cycle extension was done at 72°C for 5 min.

2.2.4.2d: **Identification of the PCR products**

Following amplification, 10µl of the PCR product was analysed on a 2.5 % agarose gel in Tris acetate EDTA (TAE) buffer using a 1 Kb ladder (Gibco) as a molecular weight marker. Amplification products were visualised by ethidium bromide. Samples amplifying at least one of the two bands (either POL or LTR) were considered positive for MVV RNA (expected size of product on the gel). The identities of the PCR products were confirmed by hybridisation with MVV specific probes.

2.2.4.3: **Analysis of DNA by blotting and hybridisation**

2.2.4.3a: **Southern transfer by alkaline method**

After the PCR products were separated on electrophoresis gels, the amplified DNA

bands were blotted to membrane (GeneScreen Plus, NEN) by capillary action using alkaline transfer method performed as described in Appendix. The membrane filters were air-dried.

2.2.4.3b: **Probes labelling systems**

Probes labelling with digoxigenin

Two ologonucleotide probes were used to identify PCR-amplified MVV POL and LTR sequences (Table 2). A digoxigenin (DIG)-based labelling and detection system was employed (Boehringer Mannheim). Probes were 3'-end labelled with DIG-11-ddUTP using terminal transferase. Briefly, the reaction mixture consisting of 5µl of TdT buffer, 3 µl of 5 mM $CaCl_2$, 2 µl of 100mM dATP, 2 µl DIG-ddUTP (1nmol/µl), 1 µg of probe, 1 µl of TdT (25u/µl) and 24 µl of H_2O was incubated at 37°C for 2hrs, diluted by adding 75 µl of H_2O and then 1 µl of Glycogen (20mg/ml) and 200 µl of ethanol were added and placed on ice for 15 min. Next, the reaction mixture was spun down at 10000 x g for 15 min at 4°C. The pellet was washed with 75% ethanol and then air dried. Finally, the pellet was dissolved in 20 µl of sterile H_2O to give a concentration of 50 ng/µl and stored at -20°C. Pre-hybridisation (in 5x SSC, 0.1% w/v N-laurylsarcosine, 0.02% SDS and 1% blocking reagent) was carried out at 65°C for 1 hr and followed by hybridization in pre-hybridization solution with 2µl (50ng/µl) of labelled probe at 65°C for 2 hrs. Bands were visualised by a colorimetric method according to the DIG Nucleic Acid Detection standard protocol (BoehrigerMannheim).

Probe labelling with ^{32}P

Probes were 5'-end labelled with ^{32}P ATP (Amersham) using polynucleotide kinase. Briefly, the reaction mixture consisting of 2µl of PNK buffer, 1 µl (60-100ng) of probe, 2 µl ^{32}P ATP (10 µci/µl), 1 µg of probe, and 14 µl of H_2O, was incubated at 37°C for 30 minutes and stopped by adding 80 µl of TE buffer (pH 8.0). The reaction mixture was added to the top of a Nick Column (Pharmacia), which was pre-treated with 1 ml of TE buffer (pH7.4). After washing with 400µl of TE buffer, the incorporated ^{32}P-probe was eluted with 400µl of TE buffer. The radioactivity

incorporation was measured. Pre-hybridization was carried out at 65°C for 1 hr and followed by hybridization in pre-hybridization solution with labelled probe, 50 µl of herring sperm DNA (10mg/ml) overnight at 65°C. Blots were washed twice with 2x SSC at room temperature and then washed twice at 60°C (15 minutes/each) with 1% SDS in 2x SSC, followed by washing with 0.1x SSC for 15 minutes. After each wash, the blot was monitored for background. After the final washing, the blots were wrapped in plastic wrap and exposed to x-ray film (Kodak).

2.2.4.4: **Cloning of MVV POL PCR product**

2.2.4.4a: **Preparation of PCR product for ligation.**

PCR product was gel-purified by excising a band of the predicted size from a low melting agarose gel following electrophoreis and recovered by Geneclean (BIO 101 Inc) as described in appendix. Briefly, 1µl Klenow polymerase (2u/µl, BoehrigerMannheim) was added into PCR product mixture and incubated at 37°C for 15 minutes and then 100 µl of TEB (pH8.0) and 100 µl of chloroform were added and mixed gently. After centrifugation (13000 rpm, 1 minute), the liquid phrase containing PCR product was collected. The PCR product was then gel-purified by running a 1% low melting agarose gel and recovered by Geneclean II kit (BIO 101 Inc.). Concentrations were estimated by electrophoresis of sample dilution on 1 % agarose gel containing 0.5 µg/ml ethidium bromide.

2.2.4.4b: **Ligation of PCR product into pCR-Script^{TM}SK(+) cloning vector**

PCR-Script^{TM}SK(+) cloning vector (Stratagene) was used to clone the PCR product according to manufacture's instruction. Ligation reactions were set up at molar ratios approximately 100:1 (Klenow treated PCR product: cloning vector). 10 ng pCR-Script^{TM} SK(+) cloning vector was used for each reaction. Ligation reactions were incubated for 2 hours at room temperature and heated to 65°C for 2 minutes. After cooling on ice, hall of each ligation was used to transform supercompetent *E.coli* cells (Stratagene). Transformants were selected by plating onto LB agar containing Ampicillin (20µg/ml), Methicillin (80 µg/ ml) and X-gal (Appendix). White colonies were picked and inoculated into a fresh X-gal/Amp./Meth. plate and placed into

falcons containing 1 ml LB/20μg/ml Amp./80 μg/ ml Meth, and then incubated overnight at 37°C with shaking (250rmp). Plasmid mini-preps were prepared by using Qiagen Plasmid Spin kits (Qiagen) according to manufacturer's instructions. Purified plasmid DNA from 1ml of culture was re-suspended in 10 μl of water. 4 μl samples were electrophoresed.

CHAPTER 3

MVV VIRAL BURDEN AND REPLICATIVE STATUS IN ALVEOLAR MACROPHAGES OF SHEEP NATURALLY INFECTED WITH MAEDI-VISNA VIURS

3.1: **Introduction**

Maedi-visna virus (MVV), a lentivirus, is a cause of inexorably progressive chronic inflammatory disorders in infected sheep including a chronic interstitial lung disease which is similar to that observed in human immunodeficiency virus type 1 (HIV-1) infection. Thereby, MVV may be considered to be a useful model system for studying AIDS pathogenesis (Carey and Dalziel 1993). As with lentiviruses of other species, MVV infection of sheep is persistent despite the continued maintenance of a strong immune response by the host (Narayan *et al.* 1982).

It is clear that chronic lymphocytic interstitial pneumonia (LIP) is the main clinical and pathological feature of MVV infection in sheep (Narayan *et al.*1985, Lairmore *et al.*1986, Brodie *et al.*1992). It is believed that MVV-infected AM are involved in the induction of proliferation of lymphocytes in the lung interstitium giving rise to an abnormal immune response through the secretion of cytokines (Gendelmam *et al.*1985, Narayan and Clements 1989, Woodall *et al.*1997). *In vitro*, MVV has been shown to infect alveolar macrophages productively. *In vivo*, MVV replication is rarely observed. AM bearing MVV capsid protein have been found at the periphery of the lesions (Brodie *et al.*1995) indicating the replication of MVV in AM may be major factor contributing to chronic inflammatory lesions in maedi (Luján *et al.*1994). But, the absolute viral load in AM and its correlation with disease onset and replicative status is poorly understood, although it is undoubtedly important for understanding the mechanism involved in the disease process.

The purpose of this study therefore was to determine the load of MVV DNA in AM compared with blood monocytes using quantitative competitive polymerase chain reaction (QC-PCR). To evaluate the MVV status in AM collected freshly from naturally infected sheep with or without histopathological evidence of lesions in the

lung, viral RNA was detected in these AM by RT-PCR and were compared with those obtained from short-term culture with stimulation by phorbol-12 myristate 13 acetate (TPA). Furthermore, the relationship between virus burden and replicative status in AM and the histopathology of the lung was assessed. To estimate MVV burden in infected cells, a QC-PCR was developed to quantify viral nucleic acid in the cells of naturally infected sheep. This technique involves co-amplifying a fixed concentration of sample DNA and graded amount of competitive template bearing the same primer recognition sequences as target DNA but containing an internal deletion such that its product is distinguishable in size from the target DNA product after amplification. The validation of the assay was subsequently accomplished using DNA isolated from AM and peripheral blood mononuclear cells (PBMCs) obtained from MVV serological-positive sheep.

CHAPTER 3:

Material and methods

3.2.1: **Animals**

Twelve MVV-infected sheep were studied at postmorten. The diagnosis of MVV infection was assessed by the agar gel immunodiffusion test (AGID) (Cutlip *et al.*1979) and the histopathological status in lung was determined according to previously established criteria (Lairmore *et al.*1986, Brodie *et al.*1992, 1995). Three uninfected sheep were evaluated as negative controls.

3.2.2: **Histopathology**

Fresh-frozen lung tissues or formalin-fixed, paraffin-embedded lung tissues were sectioned (5μ) by routine method and examined after haematoxylin and eosin staining. The distribution of inflammatory lesions in the lung with lymphocytic interstitial pneumonia (LIP) was uniform throughout the tissue, therefore, section of the cranioventral left lung lobe was considered to be representative of the entire lung.

3.2.3: **QC-PCR**

3.2.3.1: **Competitive template construction**

A 217 bp cDNA fragment (nucleotides 4448 to 4231, 217bp) of the *pol* region of MVV EV1 strain was amplified with a pair of wild-type primers POL4 and POL5 (see Table3.1) and cloned into a pCR-Script^{TM}SK(+) cloning vector (Stratagene) which was designated pPOL2. Using this wild-type fragment from pPOL2, amplification with a pair of primers POL4 and POL2DII (see Table3.1) yielded a shortened competitor fragment containing a deleted version of the original *pol* sequence (192 bp against 217bp of the full-length template) which can be amplified by the wild type primers POL4 and POL5. Primer POL2DII contains a sequence specific for a target sequence within the original POL4 and POL5 amplification product, which was synthesised on to the 3' end of the original POL5 primer sequence. POL2DII therefore acted to shift the POL5 sequence into an internal region of the original *pol* product, deleting the intervening region and the original

POL5 target sequence from the 3' end (Fig 3.1). This product was purified with a Biogene clean kit (BIO 101 Inc) and ligated in a polylinker of a pCR-Script^{TM}SK(+) cloning vector (Stratagene). The recombinant plasmid DNA (designated pΔPOL2) was purified with Qiaprep Spin plasmid kit (Qiagen) and then sequenced and quantified by spectrophotometric analysis (Fig3.1).

3.2.3.2: **Quantitative competitive PCR**

In a typical QC-PCR assay, a graded amount of competitive template DNA was added to 500ng of sample DNA. Each reaction was carried out in a 50 µl volume containing 1 U of Taq DNA polymerase (Life Technologies), 20mM (each) dNTP, 25 pmol of each primer (POL4 and POL5) and 50mM KCl and 2.5 mM $MgCl_2$ in 10mM Tris HCl. The reaction was overlaid with 30µl of mineral oil. 50 cycles of amplification were carried out in a thermal cycler (Techne PHC-1) under the following conditions: 95°C for 60s, 55°C for 60s and 72°C for 120s. Plasmid pPOL2 containing *pol* and DNA from tissue of non-infected sheep were included in each experiment as positive and negative control, respectively.

3.2.3.3: **Detection and quantitation of PCR products**

A 10 µl volume of PCR product was separated on a 2.5% agarose gel in TAE buffer. The gel was stained with ethidium bromide and visualised with UV light, then transferred to a nylon membrane (Genescreen plus^{TM}, NEN). The target DNA was detected by Southern blotting and probed with ^{32}P labelled internal DNA probe which was generated by using internal primers (POL8 and POL9). The radiolabeled hybrids were detected by autoradiography, and the intensity of each specific band was determined by densitometric analysis (Image Analyser, Millipore, UK). For each QC-PCR, a standard curve was obtained by plotting the log of the ratio of the intensity of amplified products (pΔPOL2/pPOL2)(y-axis) against the log of the pΔPOL2 known amount added into the reaction (x-axis). The original amount of MVV *pol* gene present in the sample was then calculated by interpolating the log of the ratio of the intensity of PCR products (pΔPOL2/sample) into the standard curve.

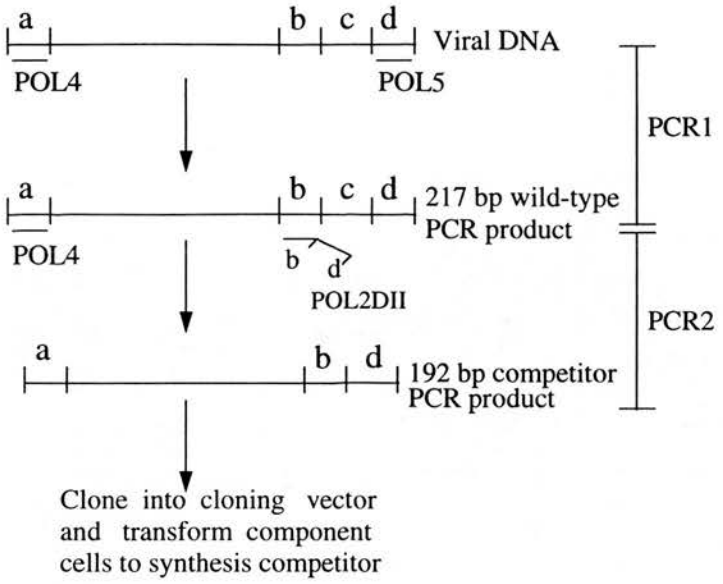


Figure 3.1 Synthesis of the deleted competitor (192 bp) from the full-length template (217 bp)

A 217 bp cDNA fragment (nucleotides 4448 to 4231, 217bp) of the *pol* region of MVV EV1 strain was amplified with a pair of wild-type primers POL4 and POL5 and cloned into a pCR-Script^{TM}SK(+) cloning vector (Stratagene) which was designated pPOL2. Using this wild-type fragment from pPOL2, amplification with a pair of primers POL4 and POL2DII yielded a shortened competitor fragment containing a deleted version of the original *pol* sequence (192 bp against 217bp of the full-length template) which can be amplified by the wild type primers POL4 and POL5 and cloned into a pCR-Script^{TM}SK(+) cloning vector (Stratagene) which was designated pΔPOL2.

3.2.4: **Alveolar macrophages**

3.2.4.1: **Study of cell morphology and cytochemistry**

The preparation of AM is described as in Chapter 2.

I. Cell morphology. Cell morphology was studied by light microscope on Leishman-stained smears. 200 cells were examined and classified by their morphological appearance.

II Cytochemistry. α-naphthyl acetate esterase activity was studied on cytocentrifuge slides and fixed formol vapor and incubated for 30 min in an α-naphthol AS-D acetate solution (Sigma).

3.2.4.2: **Surface marker analysis of alveolar macrophages**

The technique described in Chapter 2 and Index was applied using a ABC immunoperoxidase staining kit (Vector Laboratories). VPM63, a mouse McAbs anti-ovine CD32, was used to identify AM.

3.2.4.3: ***In vitro* stimulation of AM with TPA**

AM collected from six infected sheep with no histopathological evidence of lesions in the lung were cultured (10^{6}/ml) in RPMI 1640 medium, 10% foetal calf serum, and antibiotics in the presence of 1ng/ml TPA for 24hrs and harvested. Their viability was evaluated by trypan blue dye exclusion.

3.2.5: **Detection of viral nucleic acid**

3.2.5.1: **Quantitative detection of MVV DNA by QC-PCR**

Quantitative detection of MVV DNA was performed by QC-PCR, as described in 3.2.3.

3.2.5.2: **Detection of MVV RNA by RT-PCR**

Total cellular RNA was isolated using RNA easy kit (Qiagen). RNA was reverse transcribed into complementary DNA (cDNA) using Superscript II Reverse Transcriptase (Life Technologies) and random primers (Pharmacial). After reverse transcription, PCR was performed using the primers specific for MVV POL. To

assess the efficiency of RNA extraction and cDNA synthesis, PCR using ATPase specific-primers was performed as internal control. For each assay, a negative control containing only sterile water and a positive control were run in parallel. After PCR, 8 µl of aliquots of the product were electrophoresed on 2.5% agarose gel stained with ethidium bromide and photographed under ultraviolet light.

3.2.5.3: **Southern blotting and hybridisation**

Gels were Southern blotted onto Gene Screen plus membrane (NEN) by alkaline transfer according to the manufacturer's instructions. After capillary transfer, membranes were UV-cross linked and hybridised at 65°C overnight with specific ^{32}P-labelled probe for ATPase and POL. Membranes were autoradiographed with Kodak X-OMAT film.

Table 3.1. Oligonucleotide primers used in QC-PCR for MVV

Primers	Sequences (5' to 3')	No of bases(position)*
POL4	ATAGTAAATCTCATCAAGATGC	217 (4231)
POL5	TCCCGAATTTGTTTCTACCCTG	(4448)
POL2DII	TCCCGAATTTGTTTCTACCCTGAGTGTAATCCACTTGCCAAT	192
POL7	TCATTGCATCTAGAATTTG	
POL8	TGTAATCCACTTGCCAATG	

- Nucleotide positions are those from the published sequence of MVV EV-1 strain (Sargan *et al.*, 1991).

CHAPTER 3:

Results

3.3.1: **Alveolar macrophages**

AM exhibited irregular surfaces, numerous filopodia and pseudopodia characterised the cell membrane. The cytoplasm was filled with numbers of vacuoles (Fig3.2). Most AM obtained displayed esterase positive (Fig3.3A) and VPM63 (CD32) positive reaction (Fig 3.3 B). AM cultured for up to 3 weeks in RPMI/10%FCS were analysed for cell number and viability. After the second day, cell numbers decreased gradually (data not shown).

3.3.2: **QC-PCR**

3.3.2.1: **Competitive template construction**

The results for construction of DNA competitor are shown Fig.3.4 and 3.5. A fragment of 217bp from MVV *pol* was amplified with primer set POL4 and POL5 and cloned into a pCR-ScriptTMSK. The clone, designated pPOL2 was then amplified with primer POL4 and POL2DII. The expected 192 bp PCR products appeared as sharp band after get electrophoresis and ethidium bromide staining (Fig.3.5) and used as DNA competitor after cloning and purification. The PCR amplification of the competitor with primer set POL4 and POL5 resulted in a 192bp product which was distinguishable from the 217 bp of PCR-amplified viral *pol* DNA product after separation by agarose gel electrophoresis (Fig.3.5).

To compare the efficiency of amplification for the competitive and analysed templates, the ratio of the two templates over a wide range of cycles was measured (Fig.3.6). Copy number is calculated as {the amount of DNA/(molecular weight per base pair × number of base pair DNA)} × 6.023×10^{24}. 10^4 copies of pPOL2 was mixed with the same copies of competitive template and co-amplified for an increased number of cycles. Amplification was exponential for both templates after 35 cycle amplification. When both templates were amplified for 50 cycles, both

Figure 3.2 Light microscope of morphology of AM in cytocentrifuge preparation from one-day cell culture in RPMI/10%FCS

Leishmen staining (original magnification 800 x)

Figure 3.3 Cytochemistry of alveolar macrophages

A: Esterase activity of AM in cytocentrifuge preparation.
B: VPM63 McAb staining of AM in cytocentrifuge preparation. Streptavidin biotin peroxidase complex method, DAB chromogen, Mayer's hematoxylin counterstain. (original magnification 400x)

Fig.3.2

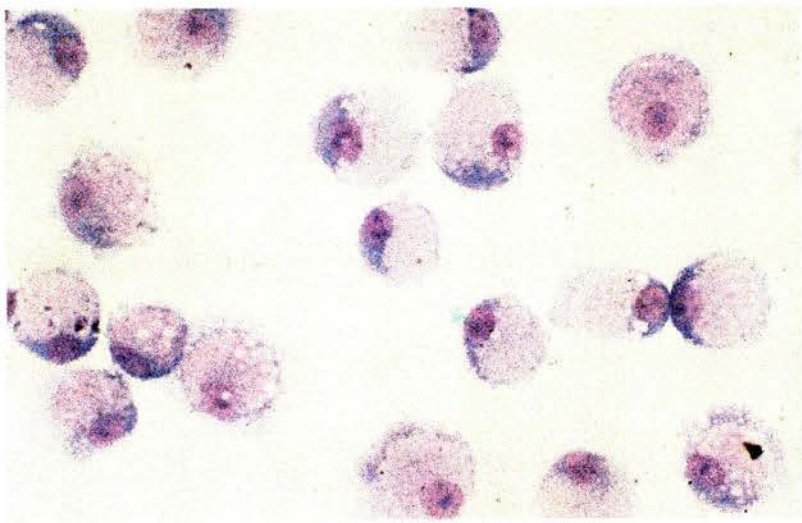
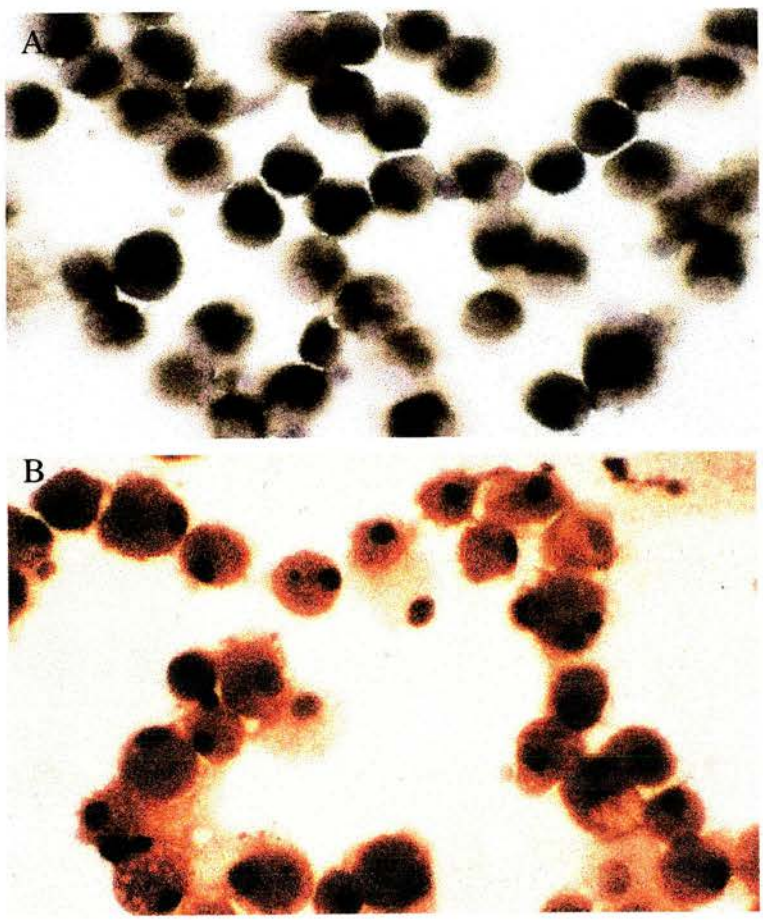


Fig.3.3



MVV POL 4231 atagtaaatggcatcaagatgc**tcaa**tcattgcatct**a**gaatttg**ac**ataccaagaa**ca**g 290

Competitor 1 atagtaaatggcatcaagatgc**agtg**tcattgcatct**g**gaatttg**gg**ataccaagaa**tg**g 60

MVV POL 4291 c**g**gct**c**aaga**t**atagtgcaacaatgtgaa**a**tatgtcaagaaaataaa**atg**ccta**at**acaa 4350

Competitor 61 c**a**gct**g**aaga**c**atagtgcaacaatgtgaa**g**tatgtcaaggaaataaa**cta**ccca**gc**acaa 120

MVV POL 4351 **t**gaga**a**ggaagtaa**c**aa**g**ag**gg**gaatagatcat**t**ggcaagtggattacactca 4402

Competitor 121 **a**gagg**g**gaagtaa**t**aa**a**aga**g**gaatagatcat**g**ggcaagtggattacactca 172

MVV POL 4429 gggtagaaacaaattcggga 4448

Competitor 173 gggtagaaacaaattcggga 192

Figure 3.4 Sequence of competitive template

Nucleotide positions are those from the published sequence of MVV EV-1 strain (Sargan *et al.*, 1991). A bold letter is shown where nucleotide is different from the wild-type template.

Figure 3.5 Amplification of the competitive template with primer set POL4 and POL5

Amplification of the competitive template with primer set POL4 and POL5 resulted in a 192bp product which was distinguishable from the 217 bp of QC-PCR-amplified viral DNA product after separation by 2% agarose gel electrophoresis.

Lane 1: Wild-type/competitor PCR products
Lane 2: Competitor PCR product
Lane 3: Wild-type PCR products

Figure3.6 The effect of the number of PCR cycles on competitive amplification

A mixture of pPOL2 DNA (10^4 copies) and competitive template pΔPOL2 (10^4 copies) was co-amplified for varying cycle numbers of PCR, as indicated. The PCR amplified products were probed with ^{32}P labelled probes. Copy number = {the amount of DNA/(molecular weight per base pair × number of base pair DNA)} × 6.023×10^{24}.

A: 2 % agarose gel stained with ethidium bromide.
B: Corresponding autoradiogram. Probed with ^{32}P labelled probe

Fig.3.5

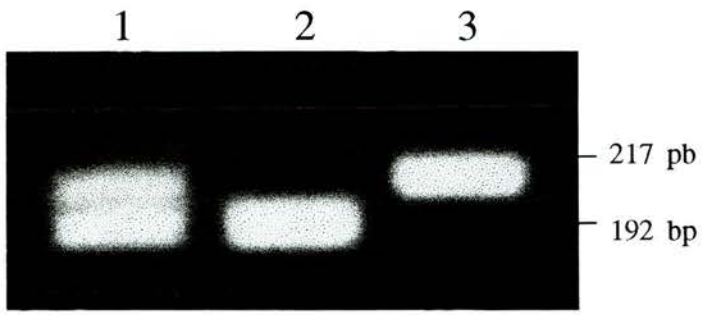
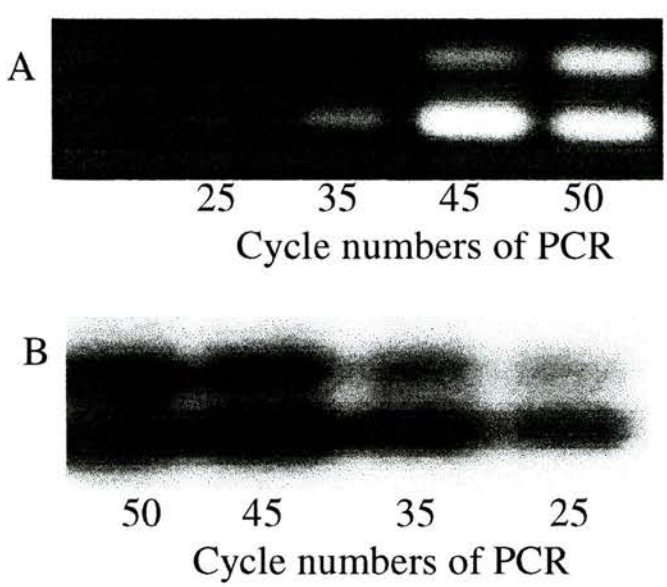

1
2
3
217 pb
192 bp

Fig 3.6


A
25
35
45
50
Cycle numbers of PCR
B
50
45
35
25
Cycle numbers of PCR

molecules gave approximately the same amount of products when the band intensity following probing and the autoradiography was analysed by the densitometry (data not shown), indicating competitive template DNA is amplified with the same efficiency as wild type template. The optimal QC-PCR cycle was then 50 cycles. Previously, it had been reported that addition of cellular DNA in each of the PCR reactions that contained two recombinant templates resulted in a decrease in amplification efficiency (Boivin *et al.* 1995). Therefore, when construction standard curve were made, all plasmids used were amplified in the presence of 500 ng cellular DNA in order to made the condition close as possible as *in vivo* condition.

3.3.2.2: **Construction of standard curve**

To obtain the standard curve, a single concentration of pPOL2 (10^5 copies) was co-amplified with a four-fold dilution of a known concentration of competitive template starting at $3x10^6$ copies (pΔPOL2). There was a progressive competition between the competitor (192 bp) and MVV cDNA (217 bp). By incorporating various amount of serially diluted competitor in PCR and measuring the corrected densitometry values, a standard curve was generated. As showed in Fig.3.7, a representative standard curve was established by plotting on a graph the $\log_{10}$ of ratio of amplified products (y-axis) against the $\log_{10}$ of the number of pΔPOL2 copies added into the reaction (x-axis) (Fig.3.7). A linear relationship was obtained between the log of the ratio of amplified products (pΔPOL2/pPOL2) and the log of the pΔPOL2 known copy numbers added into the reaction over the range of 10^2 to 3×10^6 (Fig.3.7C). The original amount of MVV pol gene present in the sample was then calculated by interpolating the log of the ratio of PCR products into the standard curve.

The lower limit for competitive quantitation with the assay has been determined as 10^2 copies per 500 ng of total cellular DNA. Serial dilutions of DNA of plasmid pPOL2 were made in AM lysates down to the equivalent of 10^2 /500 ng of total DNA. The density of the specific band (217 bp) was significantly above the background level given by negative samples. The 10^2 copies were set as the lower limit because it is technically impractical to assay accurately the numerous runs

required to quantify below the 10^2 copies in order to take account into the stochastic partitioning of low amount of DNA in a sample and that control experiment with known copy number of pPOL2 and varying pΔPOL2 fitted a standard curve well. In order to demonstrate that 10^2 copies were achieved, the 10^2 copies were diluted 10-fold into AM to 10^1 per 500ng total DNA and run five times in duplicate. The 10^1 copy per 500ng DNA was found positive 4 times out of 5. Furthermore, in the positive sample lanes, a 217 bp MVV band was present after 3 hours of autoradiography, whereas in the negative sample lanes, no MVV band could be detected even after 24 hours of autoradiography. The amount of MVV *pol* gene present in samples which were outside the range of the standard curve were reported as either $< 10^2$ or $>3x10^6$ copies per 500ng of DNA.

3.3.2.3: **Reproducibility of the QC-PCR**

As shown in Fig.3.8, when the same preparation of standard DNA was run on different days, the slopes of the linear standard curves of three separate and consecutive experiments were equivalent (correlation of SC1 and SC2 = 0.996, correlation of SC2 and SC3 = 0.993, correlation of SC1 and SC3= 0.999). This reproducibility in the slopes of standard curve is an important factor to achieve accurate quantification. Furthermore, the intra-assay variability of the procedure is low as shown by the high reproducibility of the duplicate samples (Table 3.2).

3.3 3: **Viral burden in alveolar macrophages of sheep naturally infected with MVV**

3.3.3.1: **Serology and histopathology**

All of 12 infected sheep studied had serum antibodies against the MVV by AGID test. Of seropositive animals, 6 of 12 animals had histopathological evidence of lymphoid interstitial proliferation in the lung (Fig.3.9A, B). 6 of 12 animals and 3 seronegative controls had no histopatholoical evidence of lymphocytic interstitial pneumonia or opportunistic infection in the lung (Fig.3.9C).

Figure 3.7 Construction of a standard curve

A: 2 % agarose gel stained with ethidium bromide.

Lanes 1-2: DNA positive control obtained from plasmids pPOL2 and pΔPOL2 respectively.

Lanes 3-9: Four-fold dilution (starting at $3x10^6$) of competitive template respectively (192-bp band) co-amplified with 10^5 of wild-type template (217-bp band).

B: Corresponding autoradiogram. Probed with ^{32}P labelled probe.

C: Construction of a standard curve following densitometric analysis of specific band on the autoradiogram shown in B.

Copy number = {the amount of DNA/(molecular weight per base pair × number of base pair DNA)} × 6.023 × 10^{24}.

Fig.3.7

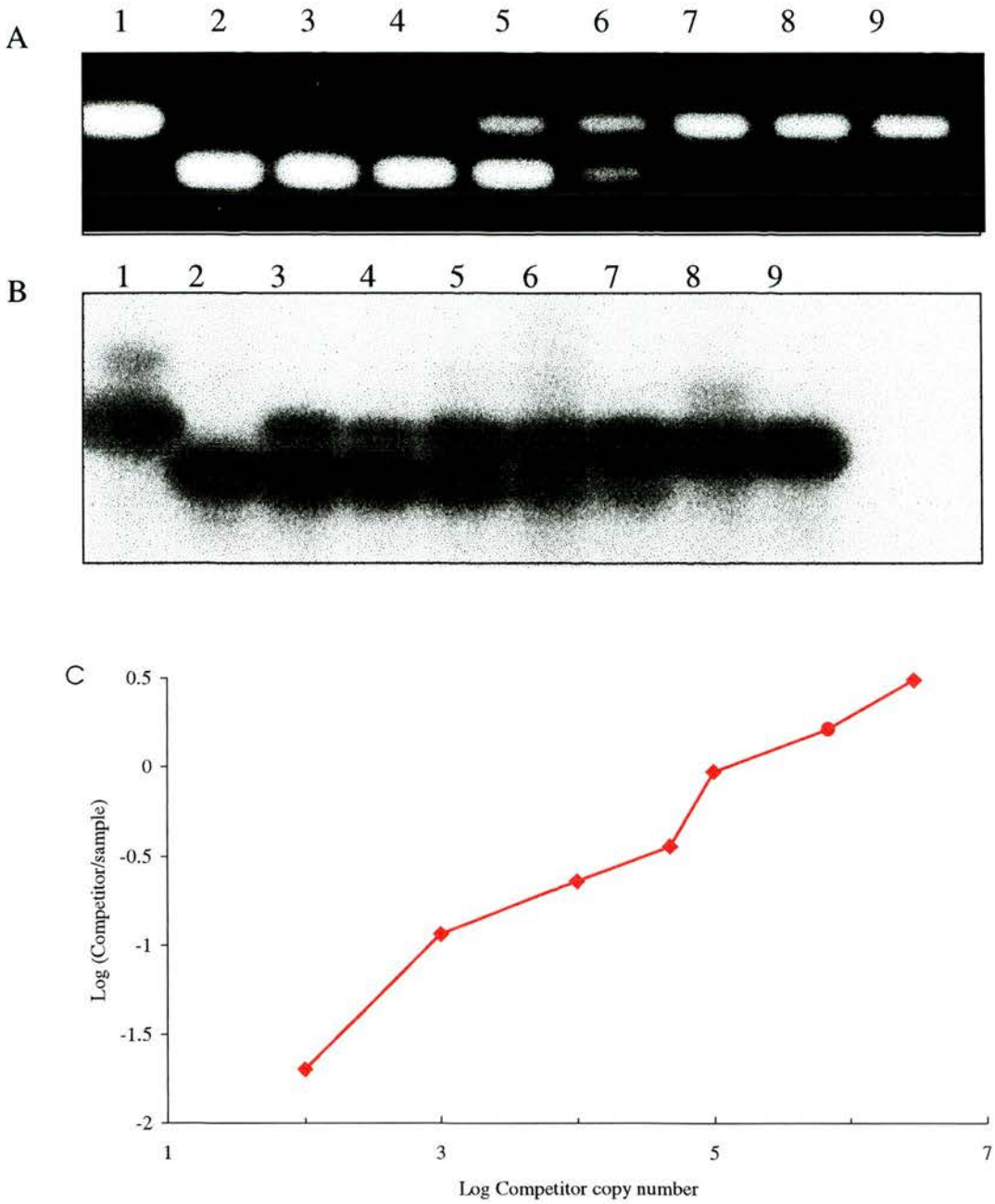

A
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8 9
C
0.5
0
-0.5
-1
-1.5
-2
Log (Competitor/sample)
1
3
5
7
Log Competitor copy number

Table 3.2 Intra-assay variability

Sample Nos.	1	2	3	4	Mean	SD
A188	0.985	0.605	0.720	1.120	0.875	0.236
A115	3.451	2.620	4.149	3.80	3.510	0.655
A011	3.400	1.921	2.330	2.750	2.725	0.63
A006	1.507	1.477	1.430	1.690	1.517	0.266
A163	<0.041	<0.041	<0.041	<0.041	<0.041	_

Five samples were run four times with different labelled probes and standard DNA. The means were calculated on five samples. The samples A163 had amount of viral DNA <0.041fg and was not included in the statistical analysis.

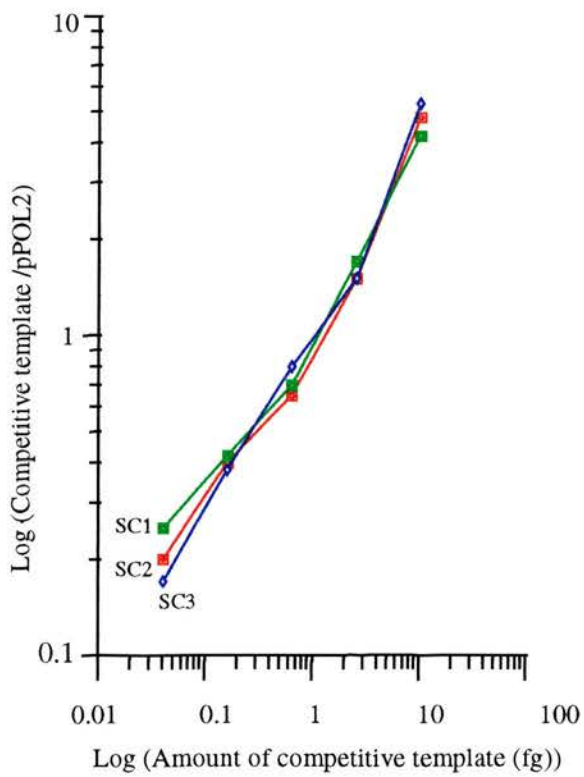


Figure 3.8 Three different Standard curves (SC) from three consecutive experiments were graphed together

Figure 3.9 Histologic sections of the cranioventral left lung lobe from sheep naturally infected with MVV

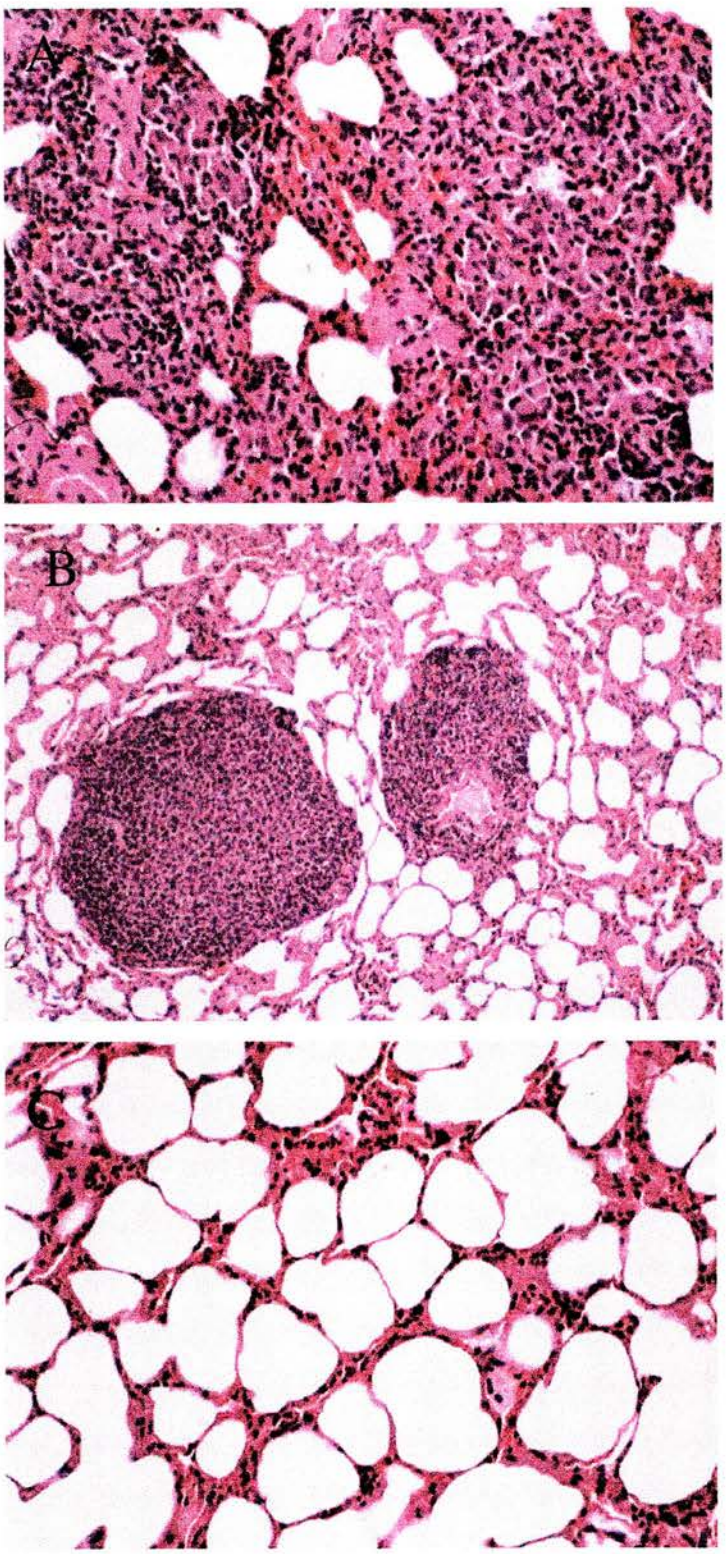
Typical histological lesions of lymphoid interstitial pneumonia in a sheep naturally infected with MVV.

A: interstitial fibromuscular proliferation.

B: follicular proliferation.

C: histologic section of lung without histopathological evidence of lesions (hematoxylin and eosin stain, original magnification 800x).

Fig.3.9



3.3.3.2: **Virus load in AM and monocytes from lung and blood**

3.3.3.2a: **Detection of viral DNA**

To assess whether viral DNA is detectable in AM and monocytes isolated from infected sheep with or without histopathological lesions in the lung, amplification of MVV DNA was performed using a pair of primers that corresponds to a conserved region of MVV POL gene. MVV DNA was detected in 12 out of 12 samples in AM, in 11 out of 12 sample in blood monocytes collected from sheep naturally infected with MVV with or without histopathological lesions in the lung. No viral DNA was detected in AM from two MVV seronegative and one SPF lambs. Representative analysis of amplified *pol* sequences by PCR is shown in Fig.3.10.

3.3.3.2b: **Virus burden in AM and blood monocytes**

Viral DNA load in AM and PBM was assessed by QC-PCR. A representative animal sample is shown in Fig 3 11. Virus DNA was detected in AM from all animals (n=9) with a median copy 319430 per 500ng cellular DNA (range from <1195 to 1032600). In PBM, infected animals (n=9) with median 36195 per 500ng cellular DNA, range from 1195 to 11956). Over the range of results obtained (10^2 to 10^6 copies per 500ng), the QC-PCR has an intra-assay variability 23% and interassay variability of 48%. The MVV DNA copy number per 500ng cellular DNA was higher in AM than PBM (Fig 3.12A). There is significant difference in viral DNA load between AM and PBM when the animals were compared overall ($P<0.05$, Mann-Whitney nonparametric statistics). Since the complexity of the diploid sheep genome is 3.0×10^9 bp, one cell contains approx.3pg DNA, therefore, 500ng of DNA could represent 160,000 cells.

3.3.3.2c: **The levels of viral DNA load in AM was compared to the histological lesions in lung**

To determine whether viral load changes differed in relation to the histological lesions in the lungs, twelve sheep naturally infected with MVV were divided into two groups according to histologic findings in the lungs. The viral DNA load in AM from both

Figure 3.10 Representative analysis of amplified *pol* sequences by PCR

Cells were isolated as described in Material and Methods. Cell lysates were analysed by PCR. The nitrocellulose filter was hybridised with ^{32}P labelled internal probe.

Lane 1: Positive control (DNA from plasmid containing *pol* sequences).
Lane2: Negative control (DNA from uninfected sheep).
Lane 3 through5: DNA from AM of MVV infected sheep with lesions in the lung.
Lanes 6and 7: DNA from AM of MVV infected sheep without lesions in the lung.

Figure 3.11 Representative quantitative analysis of viral DNA from AM by QC-PCR

AM were isolated as described in Material and Methods. Cell lysates were analysed by QC-PCR. $2x10^6$ cell equivalents were used in each reaction. PCR products were separated in 2% agarose and detected by Southern hybridisation.

A: AM obtained from MVV infected sheep with lesions in the lung.
B: AM obtained from MVV infected sheep without histological lesions in the lung.

Lane1: 500ng of sample DNA.
Lane 2: pΔPOL2 control.
Lanes 3-9: Four-fold dilution of pΔPOL2 starting with 3×10^6

Fig.3.10

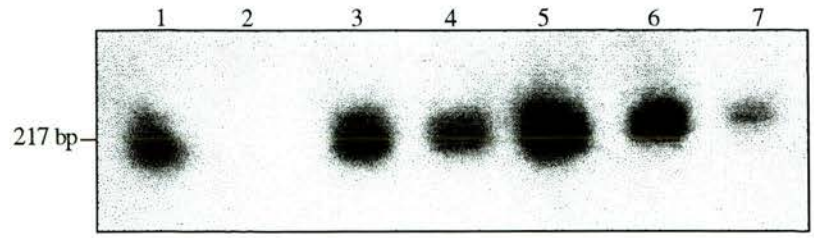


Fig.3.11

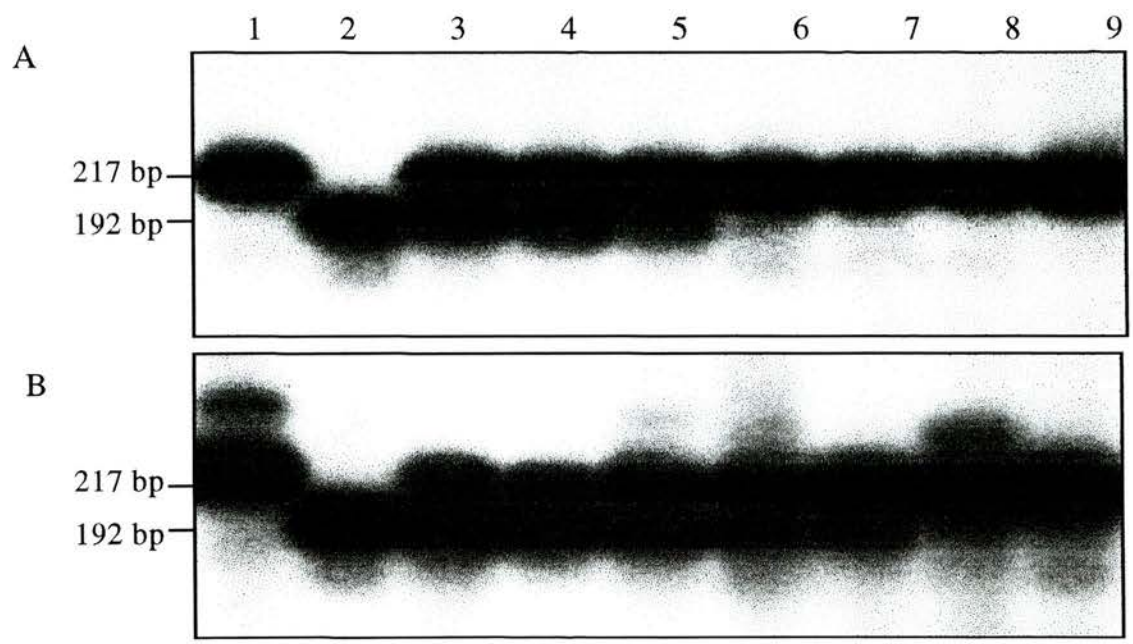


Figure 3.12 Virus load in AM and peripheral blood monocytes

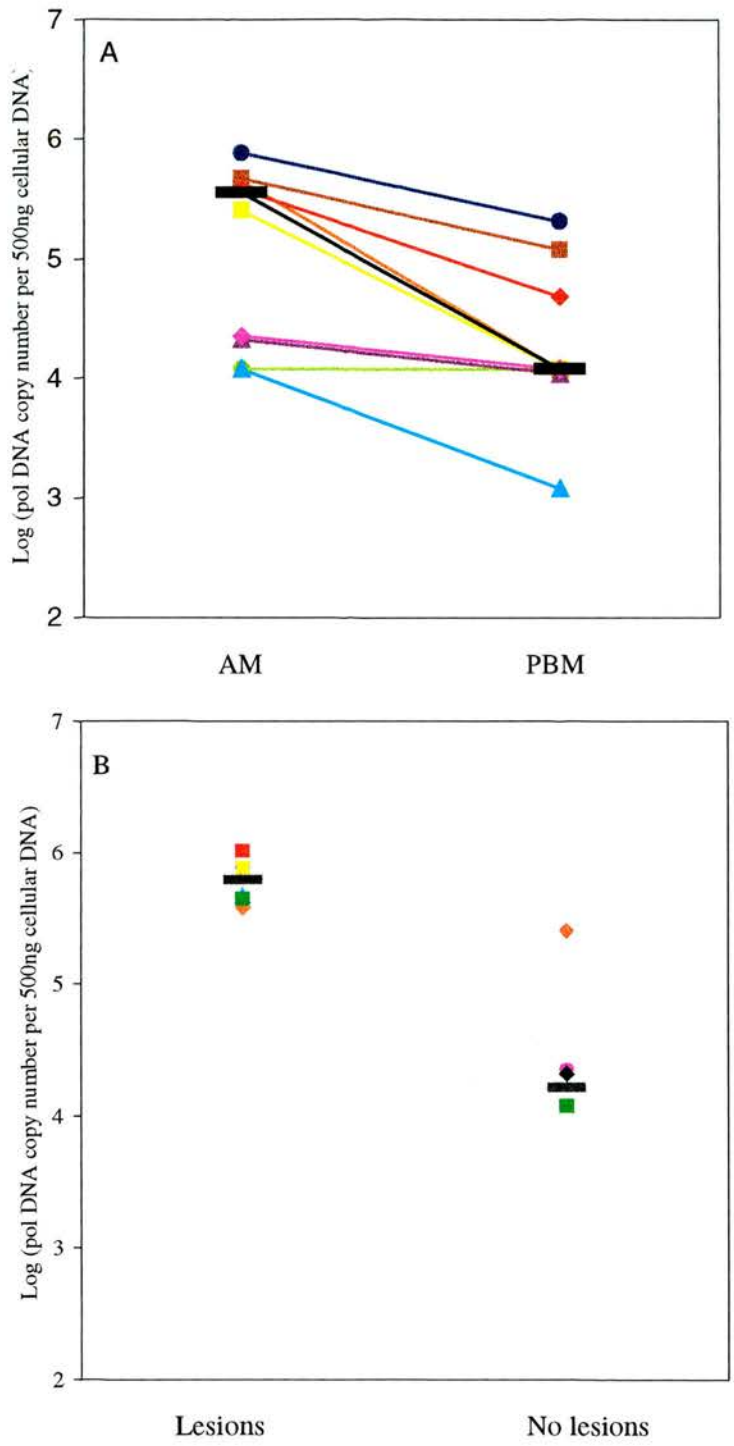
A: Quantification of MVV *pol* DNA copy number in AM and PBMC from MVV–infected sheep.

Paired results of *pol* DNA copy number per 500 ng cellular DNA of alveolar macrophages (AM) and peripheral blood monocytes (PBM) from each animal. Horizontal bars indicate median copy number. Since the complexity of the diploid sheep genome is 3.0×10^9 bp, one cell contains approx.3pg DNA, therefore, 500ng of DNA could represent 160000 cells. There is significant difference in viral load between two groups ($P<0.05$, Mann-Whitney nonparametric statistics).

B: Quantification of MVV *pol* DNA copy number in AM from MVV-infected sheep.

Paired results of *pol* DNA copy number per 500 ng cellular DNA of AM obtained from MVV-infected sheep either with (n=6) or without (n=6) pulmonary lesions associated with MMV infection. Horizontal bars indicate median copy number. There is significant difference in viral load between two groups ($P<0.05$, Mann-Whitney nonparametric statistics).

Fig.3.12


A
Log (pol DNA copy number per 500ng cellular DNA)
7
6
5
4
3
2
AM
PBM
B
Log (pol DNA copy number per 500ng cellular DNA)
7
6
5
4
3
2
Lesions
No lesions

group animals is recorded in Fig.3.12B. Among these MVV infected animals used in the experiment, lung lesions were observed in the cases showing higher level of viral DNA load in AM whereas no lung lesions were noted in the cases showing lower level of viral DNA load in AM. As shown in Fig.3.12B, in AM of animals with histopathological lesions in the lung the median MVV DNA load was 620120 per 500ng cellular DNA (range 38293 to 103260). Conversely, in AM of animals without histopathological lesions in the lung the median MVV DNA load was 65755 per 500ng cellular DNA (range <1195 to 25594). There is significant difference in viral load between two groups ($P<0.05$, Mann-Whitney nonparametric statistics).

3.3.4: **Relationship between virus replicative status in AM and histopathology of the lung**

To determine the relationship between MVV replicative status and the histological lesions in the lungs, nine sheep naturally infected with MVV were divided into two groups according to histologic findings in the lungs. To detect viral RNA in AM, RT-PCR was performed using a primer corresponding to a conserved region of *pol* gene. PCR products were hybridised with ^{32}P-labeled internal probes. To assess the efficiency of RNA extraction and cDNA synthesis, PCR using ATPase specific-primers was performed as internal control. The procedure was performed twice to test the reproducibility of the assay.

3.3.4.1: **Detection of MVV RNA in AM by RT-PCR**

To detect viral RNA in AM, amplification of MVV cDNA was performed using a pair of primers that corresponds to a conserved region of POL gene. MVV RNA sequences were detected in 6 of 6 AM samples collected from MVV-infected sheep with histopathological lesions in the lung. Representative analysis of amplified *pol* sequences by RT-PCR. is shown in Fig.3.13. In contrast, viral RNA was not detected in fresh AM collected from three sheep naturally infected with MVV without histopathological lesions, and no samples from two MVV seronegative sheep and one SPF lambs. Therefore, it can be considered that MVV is not in active replication in AM from lungs without histological evidence of lesions, indicating that detectable viral replication is a factor in the progression of lymphocytic interstitial pneumonia.

Interestingly, it was found that MVV RNA was undetectable in AM freshly isolated from infected sheep without lung histological lesions whereas viral RNA could be detected in lymph node and spleen from the same animals. Fig. 3.14 and 3.15 show the results of amplification of viral RNA from AM and lymphoid node chorio-mediastinal lymph node (CMLN) of two naturally infected animals without histopathological lesions in the lung using *pol* primers, respectively. MVV RNA is undetectable in AM from the lung without histopathological lesions but detectable in lymphoid nodes (Fig.3.14). Results were similar when another primer pair gag1/gag2, which amplifies a conserved region of the *gag* gene, was used (Fig.3.15). These findings indicate that lymphoid tissue is important reservoir of virus in MVV infection. Results in spleen were similar to lymph nodes when primer pair *pol* used (Fig.3.16).

3.3.4.2: ***In vitro*** **stimulation of AM**

To determine whether MVV RNA could become detectable in AM isolated from sheep without histological lesion in the lung, AM were cultured *in vitro* with stimulation with TPA (RNA expression stimulator). Initially various concentrations of TPA were investigated to optimise the stimulating effect on viral RNA expression. After removal of non-adherent cells from PBMC preparations, the adherent cells (monocytes) were treated with TPA at four different concentrations, cultured at 37°C for 48 hrs and then analysed by RT-PCR using *pol* and LTR primers. Results presented in Fig.3.17 indicated that TPA at a concentration of 1ng/ml or more appeared to be very effective in stimulating viral RNA expression in adherent cells. Verification of identity was initially made by radioactive Southern blotting using a POL probe. With respect to TPA cytotoxicity, TPA at a concentration of 1ng/ml was chosen for use through the experiment. To determine the minimum time required for effective stimulation, adherent cells. derived from PBMC from MVV infected sheep were treated separately with TPA at a concentration of 1ng/ml for different times during period from 0 to 72 hrs. Results shown in Fig 3.18 indicated that a minimum of 48 hrs of treatment with TPA is enough to stimulate viral DNA expression in adherent cells to a much greater extent.

In TPA treated cells, viral RNA was expressed at a more rapid rate and higher level that in non-treated cells on the basis of electrophoretic band density on gels stained by ethidium bromide.

AM were then cultured *in vitro* with stimulation with TPA (1ng/ml) for 48 hour and immunocytochemistry using anti-p25 McAb and RT-PCR using POL primers were performed on AM before and after stimulation with TPA to identify MVV replication. The results are shown in Fig3.19 and Fig3.20, before culture and stimulation with TPA, viral p25 antigens and RNA were not detected. However, after short-term culture of these AM and *in vitro* stimulation with TPA, Viral RNA became detectable in these AM and p25 positive cells were found. These results show TPA stimulation activated MVV RNA expression in AM from sheep without lung lesions indicating MVV is latent in these AM but replicates after *in vitro* stimulation.

Figure 3.13 Representative analysis of amplified pol sequences in freshly isolated AM from MVV infected sheep with lung lesions by RT-PCR using ^{32}P-labelled probes

A: POL

B: ATPase

Lane 1: Positive control.

Lane 2: Negative control.

Lanes 3 -4: AM obtained from MVV infected sheep with lung lesions.

Lanes 5-6: AM obtained from MVV infected sheep without lung lesions.

Figure 3.14 The results of amplification of viral RNA from AM and lymph node (CMLN) of two naturally infected animals without histopathological lesions in the lung using POL primers and ATPase primers in the same tube

Lane 1: positive control.

Lanes 2 and 3: lymph nodes.

Lanes 4 and 5: AM.

Lane 6: negative control.

Lane7: 1kb ladder marker

Fig. 3.13

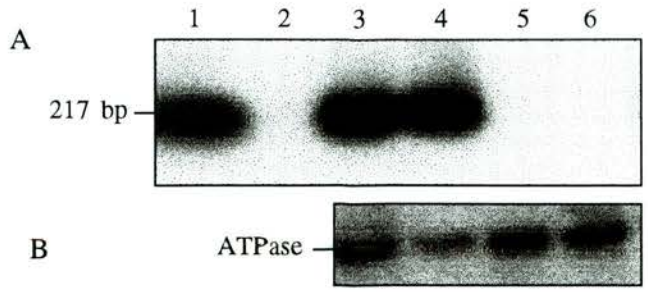


Fig.3.14

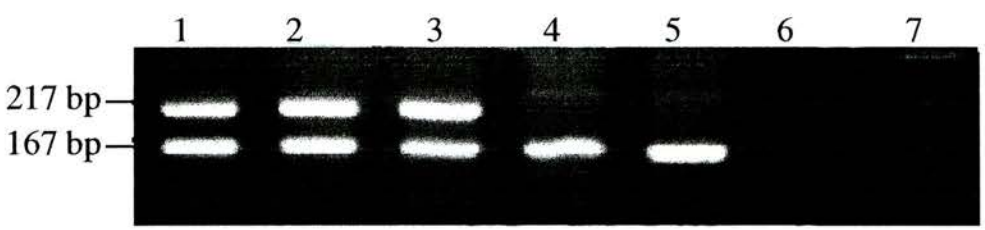


Figure 3.15 The results of amplification of viral RNA from AM and lymphoid node (CMLN) of two naturally infected animals without histopathological lesions in the lung using gag primers

Lane 1: positive control.

Lanes 2 and 3: lymphoid nodes.

Lanes 4 and 5: AM.

Lane 6: negative control.

Lane 7: 1kb ladder marker.

Figure 3.16 The results of amplification of viral RNA from AM and spleen of two naturally infected animals without histopathological lesions in the lung using POL primers and ATPase primers

Lanes 1 and 2: spleen.

Lanes 3 and 4: AM.

N: negative control.

P: positive control.

MW: 1kb ladder marker

Fig.3.15

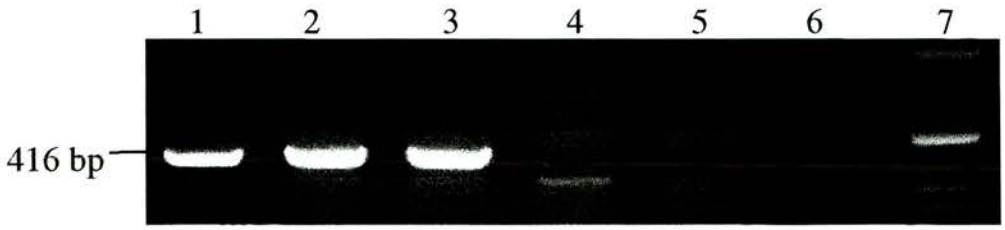


Fig.3.16

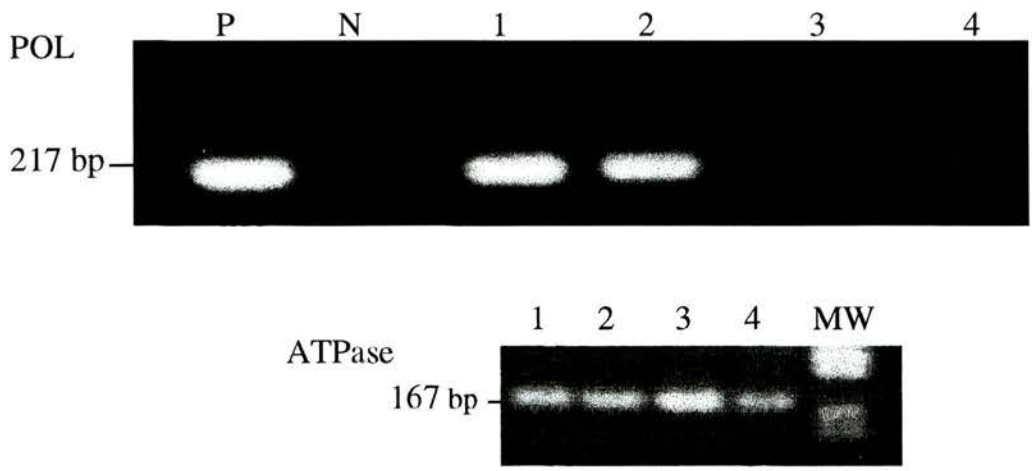


Figure 3.17 Establishment of a minimum concentration of TPA giving the greatest stimulating effect on viral RNA expression in adherent cells

Blood monocytes isolated from MVV-infected sheep were exposed to TPA at concentration of 100, 10, 1, 0.1ng/ml on the day 0 (day of isolation) harvested at 48 hours after infection. Lysates were analysed by RT-PCR with primers *LTR* (which amplifies a 156/116 bp region of LTR), and *pol* (which amplifies 217 bp region of *pol*) $1x10^6$ cells were used in each reaction. PCR products were separated in 2% agarose and stained with ethidium bromide.

A: POL primers. B: LTR primers.
Lanes 1: 1kb DNA ladder.
Lane 2: Purified MVV DNA.
Lane 3: negative control.
Lanes 4 -7: 100, 10, 1, 0.1ng/ml of TPA, respectively.
Lane 8: non-TPA treated cells.

Figure 3.18 Comparison of treatment time with TPA

Blood monocytes isolated from MVV-infected sheep were incubated with TPA at concentration of 1ng/ml and harvested at 0, 24, 48 and 72 hours after incubation with TPA. Lysates were analysed by RT-PCR with primers *pol* (which amplifies 217 bp region of *pol*). $1x10^6$ cell were used in each reaction. PCR products were separated in 2% agarose and stained with ethidium bromide.

A: POL primers. B :ATPase primers:
Lanes 1: 1kb DNA ladder,
Lane 2: purified MVV DNA,
Lane 3: negative control,
Lanes 4-7: 72, 48, 24, 0 hrs after treatment, respectively.

Fig.3.17

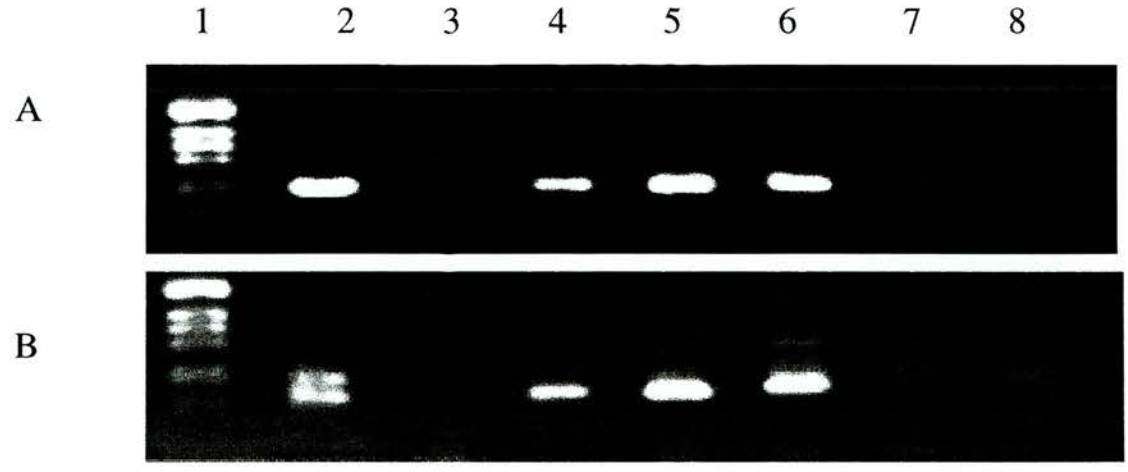


Fig.3.18

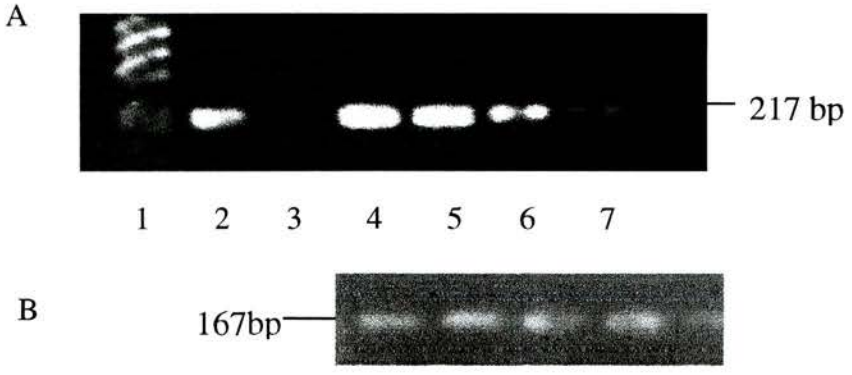


Figure 3.19 Representative analysis of induction of MVV replication in AM from infected sheep without histological lesions in the lung. Probed with ^{32}P labelled internal probe

A: Detection of MVV *pol* gene by RT-PCR.

B: ATPase

Lanes 1, 3, 5: AM treated with TPA (1 ng/ml).

Lane 2, 4, 6: freshly isolated AM.

Lane 7: Negative.

Lane 8: Positive control.

Fig.3.19

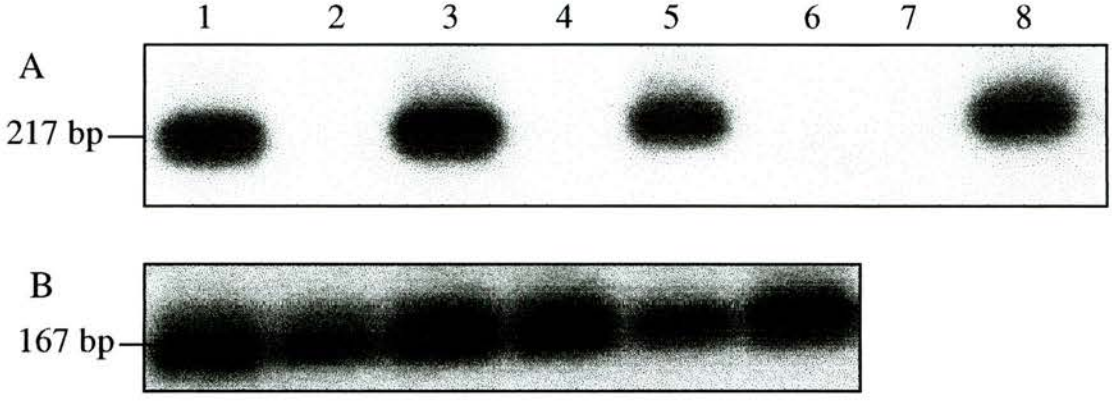
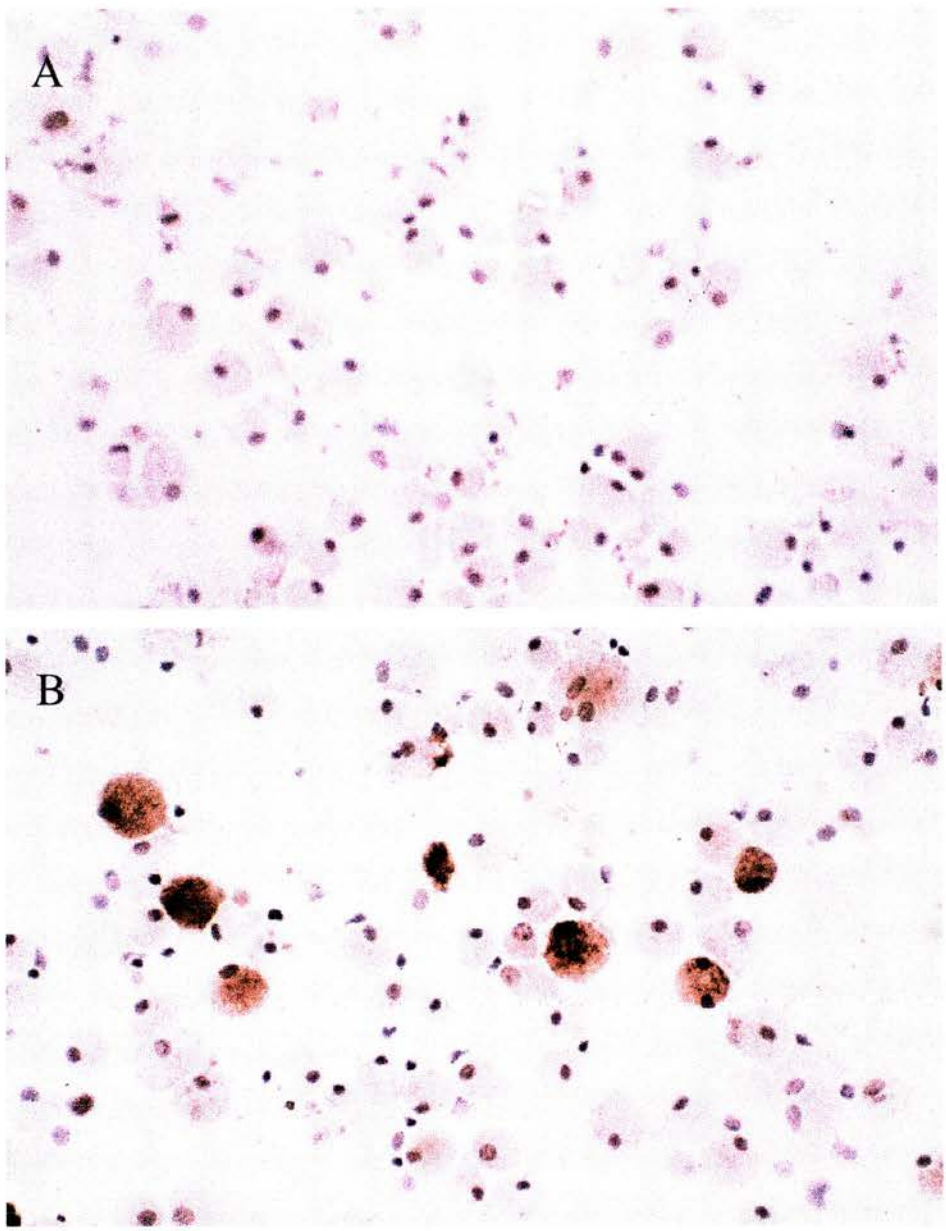


Figure 3.20 Evaluation of p25 production in AM by immunochemistry staining using ABC complex method

A: before culture and stimulation with TPA, viral p25 antigens were not detected. (Original magnification x 200 hematoxylin counterstain).

B: after short-term culture of these AM and *in vitro* stimulation with TPA (1 ng/ml), p25 positive cells were found.

Fig.3.20



CHAPTER 3:

Discussion

3.4.1: **Quantitative competitive PCR**

In this study, QC-PCR was developed for the amplification and quantification of MVV pol sequence in macrophages. The strategy for generation of the competitor construct was based of the exponential nature of PCR amplification small change in overall efficiency may be reflected in dramatic changes in the yield of PCR product which makes inter-sample comparison and quantification unreliable. It has been described that equal amplification efficiency required the competitive template to share the same primer recognition site as the target sequences to be quantified, have similar internal sequence and similar size, if the diversity in both templates length is higher that 15% (Clementi *et al.* 1994), significant differences in amplification efficiencies would occur. For this reason, internal deletion has to be reduced to the minimum necessary for obtaining discrete bands by gel electrophoresis. Based on this general concept, The QC-PCR developed here uses an internal standard DNA bearing a 25 bp internal deletion (11.5% diversity in length) which is derived from a conserved region pol of MVV The competitor template amplicans can be readily distinguished from that of wild-type template by gel electrophoresis. The two different templates were co-amplified in the same reaction mixture, competing for the same primers. The result shows that the internal standard DNA template was amplified with the nearly same efficiency as that of wild-type template when both the wild-type and competitive templates were co-amplified in the same reaction mixture, competing for the same primers. The relative ratio between competitor product and wild-type template product remains constant throughout amplification. The viral load in samples can be calculated by determining the initial concentration of the competitive template. When the QC-PCR developed was used to quantify viral DNA load in AM and PBM obtained from MVV serological-positive sheep, it was found to be reproducible and sensitive over range of 10^2 to 10^5 copies per 500ng of DNA. Within these limits, the intra-assay and inter-assay variabilities found to low which is sufficient to discriminate low, intermediate, and high values. Taken together, the QC-PCR described here allows not only the verification of the presence

of MVV, but also provides information about precise quantification of MVV DNA in infected hosts. This assay will be useful in studying the pathogenesis of MVV infection, and monitoring the dynamics of MVV replication *in vivo* at the molecular level.

3.4.2: **Viral burden and replicative status in alveolar macrophages of sheep naturally infected with MVV**

The load of MVV in AM and PBM and virus replicative status in AM from MVV infected sheep was examined. The level of viral load in AM from sheep with lung lesions associated with MVV infection was higher than that from sheep without lung lesions as determined by QC-PCR. Therefore the absolute viral load in AM may reflect the pathologic manifestation in the lung. In this study, the PCR used detects all replicate competents of the virus and dose not distinguish between replicate-competent (integrated) and unintegrated viral DNA. The latter may be present in the cytoplasm of infected cells and may constitute a labile inducible reservoir (Bukrinsky *et al* 1991). As suggested by Bukrinsky *et al* (1991), most of the viral DNA detected by PCR is likely to unintegrated reservoir. Therefore the viral load detected in this study can not directly reflect the frequency of latently infected AM. It is clear that integration of MVV DNA is necessary for productive infection (List *et al.* 1997).

The results in this study showed that increased level of viral DNA correlated with the ability to amplify viral RNA by RT-PCR and lung lesions. In this study using RT-PCR, viral RNA was found in freshly isolated AM from sheep with histological lesions in the lung, In contrast no viral RNA was detected in freshly isolated AM from infected sheep without lung lesions, but after *in vitro* stimulation, MVV RNA was observed in these AM (Fig.3.13 and 3.14). These results may explain the reasons why no viral antigens p25 could be detected in AM (Fig3.20). Therefore, it can be considered that MVV is not in active replication in AM *in vivo*, collected from infected sheep without histological evidence of lesions in the lung. AM are latently infected by MVV *in vivo*, and at resting conditions are not a site for viral

replication, therefore AM may act ac a latent reservoir for the virus. This leads to hypothesis that MVV may reside within AM in a latent state or restricted replication as a provirus integrated within host genomic DNA and the replication of MVV in AM *in vivo* may require factors which are able to activate viral replication. This could be related to still unknown mechanisms that inhibit replication of MVV in AM *in vivo* and can be removed after *in vitro* incubation. It has been shown that any differentiation stimuli can regulate viral replication and control of viral persistence in macrophages (O'Brien *et al.*1994) and that cellular factors influence permissiveness of macrophages to productive infection (Gendelman *et al.*1990c) and underscore differences between macrophages. Of these factors, cytokines produced during host immune response to viral infection particular merits consideration. For example, in the case of HIV, low level of interferons or other negative regulatory factors may be induced in response to HIV infection in monocytes (Gessani *et al.* 1991). *In vitro* systems in which HIV-1 infected macrophages are maintained in culture and exposed to different stimuli have demonstrated that HIV-1 re-enters the replicative cycle after exposure of infected cells to a number of cytokines including IL-1, IL-6, GM-CSF (for review, see Chapter 1). As a result, cytokines may have a similar crucial role, at least in part, in controlling MVV replication in infected cells. However, no clear explanations can be forwarded for the difference in the viral replicative status in macrophages in this compared with previous studies. The comprehension of the regulatory network between MVV and cytokines represents a basic question that awaits elucidation.

In this study, when detection of viral RNA was compared in AM, spleen and CMLN obtained from animals without evidence of pulmonary lesions in lung using RT-PCR, it was found that viral RNA was undetectable in AM but detected in spleen and lymph nodes. Thus MVV appears to actively replicate in lymphoid tissue but not in lung although activatable virus is present in lung. The difference may be due to the cell type being infected in these tissues or by the presence of local factors such as cytokines which exert a profound effect on MVV replication. In the lymphoid organs throughout the body, target cells together (with immune complex formation by

accessory cells) constitute the ideal milieu for active viral replication This finding promotes a consideration that lymphoid tissues may be efficient reservoirs of MVV and a site for viral replication, where target cells may be infected by MVV and transmitted to other tissues such as lung of the body. The localised infected target cells may results in local release various cytokines and other factors which may in turn recruit mononuclear cells including infected target cells into the inflammatory area. This dissociation between the viral detection in AM and in lymphoid tissue emphasises the importance of analysing both compartments when efficiency of any anti-lentivirus drug is tested.

In this study, the level of viral DNA load in AM from infected sheep was found to be higher than in PBM, suggesting that AM may be more permissive for MVV replication than PBM. The previous study demonstrated an increased susceptibility of *in vitro* differentiated monocyte to infection with MVV *in vitro* compared with that of freshly isolated monocytes (Gorrell *et al.* 1982, Narayan *et al.* 1988). Thus, The increased level of MVV in AM compared with that in monocytes from MVV infected sheep with lung lesions also may reflect an inherent increased susceptibility to infection. The mechanism by which differentiated macrophages are more susceptible to productive infection with MVV *in vivo* and *in vitro* is not known.

AM from MVV infected sheep without lung lesions harbour low level of viral DNA. As suggested by Schnittman *et al* (1990) using PCR virus production per cell remains stable while the number of infected cells increase with disease progression. Thus the low level of viral load may be a reflection of low number of infected AM in MVV infected sheep without lung lesions. Because of the low proportion of MVV-infected AM in the lung (in early stage of disease), it is unlikely that chronic active inflammatory changes with lymphocyte infiltration and proliferation in lung (Lairmore *et al.* 1986, 1988b, Watt *et al.* 1994) are directly related to viral infection of the cells. It is thought that lentivirus infection results in local release various cytokines and other factors which may in turn recruit mononuclear cells into the inflammatory area, thus providing the increased number of susceptible cells for viral

replication (Lairmore *et al.*1986, Zink *et al.*1990). The result of this study indicates that the viral DNA burdens in AM correlated with lung lesions of disease provides support for such mechanisms.

In conclusion, there was a significantly higher amount of MVV DNA and detectable viral RNA in AM from sheep with histopathological lesions in the lung compared to DNA levels and lack of detectable viral RNA in AM from sheep without histopathological lesions, suggesting that the level of MVV DNA and its replication status in AM from MVV infected sheep was correlated with histopathological lesions in the lung. In AM from sheep without histopathological lesions in the lung, MVV resides in a latent/ restricted replication status and AM may be a site for latent reservoir of MVV.

CHAPTER 4

ANALYSIS OF CYTOKINE mRNA EXPRESSION IN MACROPHAGES INFECTED WITH MVV *IN VIVO* AND *IN VITRO*

4.1: **Introduction**

MVV causes persistent infection in macrophages of sheep, which leads to mononuclear infiltration of various tissues. The precise mechanisms by which MVV causes damage are largely unknown, but dysregulation of expression of cytokines has been postulated as a possible mechanism of maintaining or exacerbating inflammation (Lechner *et al.* 1996, Woodall *et al.* 1997). Early studies showed that cytokines are likely to be present in complicated lentiviral pneumonia in sheep (Ellis *et al.* 1991), modulating MVV replication in AM (Ellis *et al.* 1994). A recent study extended these observations, showing that IFN-γ, IL-10, IL-1β, IL-4, IL-2 receptor mRNA was upregulated in lung tissue of MVV-infected sheep with lung lesions (Woodall *et al.* 1997). In particular, hyper-elevation of GM-CSF mRNA was found in lung tissue of MVV infected sheep with lung lesions (Woodall *et al.* 1997). TNF-α and TGF-β mRNA levels were similar to the lung tissue without lung lesions. These observations provide support for the concept that the viral infection results in the local release of various cytokines and other factors which may in turn recruit mononuclear cells into the inflammatory area, thus providing the increased number of susceptible cells for viral replication or modulate the viral replication (Lairmore *et al.*1986, Zink *et al.*1990). However, the type of cell (s) responsible for this dysregulation of expression of cytokines by MVV remains unknown. Furthermore, as demonstrated in Chapter 3, AM from MVV infected sheep without lung lesions harbour low level of viral DNA and the number of infected AM is low (Brodie *et al.* 1992). Therefore, it is unclear whether the low proportion of infected cells contributes to the dysregulation of cytokine expression observed in infected sheep (Woodall *et al.* 1997). In this context, the expression of cytokine genes was investigated in macrophages infected with MVV *in vitro* and *in vivo*. *In vitro* the macrophages were infected with MVV with low $TCID_{50}$ in order to mimic *in vivo* condition.

CHAPTER 4:

Materials and Methods

4.2.1: **Detection of cytokines mRNA by relative RT-PCR**

4.2.1.1: **Cytokine primers**

PCR primers (Table 4.1) were designed from published ovine sequences and synthesised by "Genosys" (Cambridge, England): ATPase (Shull *et al.*1985), IL-4 (Seow *et al.* 1993), IL-10 (Dutia *et al.* 1994), TNF-α (Woodall *et al.* 1997), GM-CSF (Mclnnes and Haig *et al.* 1991), IL-6 (Ebrahimi *et al.* 1995), MHC class II DQα and MHC class II DRα (Ballingall *et al.* 1992, Wright *et al.* 1994), TGF-β (Woodall *et al.* 1994b).

4.2.1.2: **Semi-quantatition of cytokine mRNA by RT-PCR**

RNA extraction and cDNA synthesis were performed as described in Chapter 2. Diluted RT reaction mixture (3 μl) was used as a template in PCR. PCR was performed in 50 μl PCR reaction mixture as described in Chapter 2 with the two different pairs of primers in the same tube. One pair of primers was ATPase and the other for cytokine analysis. The optimal number of PCR cycles was determined by a variable numbers of cycles to identify a linear range of amplification (i.e. to produce sub-saturation levels of PCR products) for each transcript. IL-6 was amplified for 32 cycles, IL-10 for 30 cycles, TNF-α for 30 cycles, TGF-β for 32 cycles and GM-CSF for 29 cycles. Eight microliters of amplification mixtures were separated by agarose gel electrophoresis. The PCR products were transferred to nylon membrane (Genescreen plus™, NEN research products, Boston, MA), and probed with ^{32}P-5-end-labeled probes. Bands were visualised by autoradiography, and band density quantified by densitometry (Image Analyser, Millipore, UK). Results were presented as the ratio of the signal from each cytokine mRNA to the signal obtained from ATPase mRNA. A plasmid containing each cytokine (generous gift of Dr C. Woodall, Edinburgh University, UK) was used as an internal standard for each cytokine. All samples were measured for each cytokine in the same batch and all the cytokine amplification was performed on the same cDNA synthesis.

Table 4.1 Sequences of PCR primers used to amplify cDNAs of cytokines and probes used to detect amplification products.

Cytokines	Primers
IL-4	TGATCCCAGCGCTGGTGTGC
	GTCTCTCAGCGTACTTGTACT
	AGCCACATGTGCTTGAACAAATTCCT(p)
IL-10	ATGCCACAGGCTGAGAAC
	GTTCACAGAGAAGCTCAG
	ACCTGCTCCACCGCCTTG (p)
TNF-α	ATGAGCACCAAAAGCATGATCC
	GAAGAGCTGGTGGCTCC
	AGGAGGTGCTCTCCAACAAAGCA(p)
GM- CSF	AGTCCTCAAGAGCATGTGGC
	GCGATCTGTGAGGTAAGCTT
	AACGACAGCACTGACACTGCTGCTGTG (p)
TGF-β	GCCCTGGACACCAACTACTG
	TCAGCTGCACTTGCGGAG
	CCTTCCGGAAGTCAATGTAGAGCTG (p)
MHC class II DRα	ACCAGTTTAAAGGCCAGTGTTACTTCA
	TAGTTGTGTCTGCACACCGTGTCCAC
MHC class II DQα	GGTGCGGTTCCTGGACACATACTTCCA
	CGTAGTTGTGTCTGCAGTACGTGTCCAC
DR/DQ	GGAACAGCCAGAAGGACTTCCTGGAGC (p)
ATPase	GCTGACTTGGTCATCTGC
	CAGGTAGGTTTGAGGGGATAC

CHAPTER 4:

Results

4.3.1: **Standardisation and evaluation of RT-PCR for detection of transcripts of cytokines**

To control the quality of each RNA preparation, cDNA preparation and PCR procedures, the RT-PCR was performed with primers specific for ATPase sequences which yield an amplification product of 167 base pairs (bp). The expected band representing ATPase sequences were obtained from all the samples evaluated. This approach allowed relative amounts of cytokine mRNA to be estimated using the ATPase signal as standard. The quantity of the total RNA for cytokine testing was first evaluated using ATPase and normalised to produce equivalent amounts of PCR products as ATPase (Fig.4.1). All samples were checked in the same test run for each cytokine. To examine further the reproducibility of the assay, RT-PCR testing for TGF-β mRNA and TNF-α mRNA were repeated from LPS-stimulated MDMs on three different days. The ratio of TGF-β mRNA to ATPase mRNA was 0.78 ± 0.20 and the ratio of TNF-α mRNA to ATPase mRNA was 0.59 ± 0.19.

4.3.2: **Cytokine gene expression by AM and blood monocytes from MVV-infected sheep**

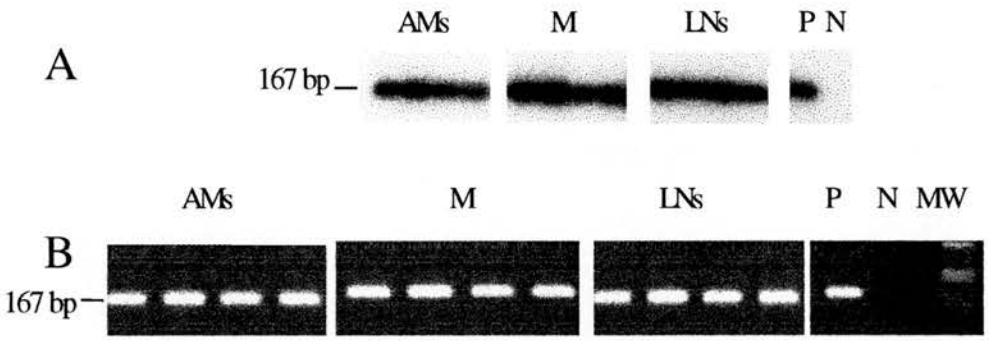
Cytokine transcription patterns in AM and PBM from MVV-infected sheep were investigated. As shown in Fig 4.2. an increase in expression of IL-6, IL-10, GM-CSF and TGF-β mRNA was observed in AM isolated from naturally infected animal (n=12) with or without histological lesions of maedi in their lungs when compared with that in seronegative controls. In PBM isolated from MVV-infected sheep (n=6) the expression of IL-6 and IL-10 mRNA was noticed and an increased expression of TGF-β and GM-CSF levels was observed when compared with these of control animals (Fig.4.3).

In addition to the result in both AM and PBM, choriomediastinal lymph node, (CMLNs) of MVV-infected sheep (n=6) contained higher level of IL-6, and IL-10 mRNA when compared with those in seronegative controls (n=3, Fig.4.4).

Figure 4.1 Detection of the housekeeping gene ATPase mRNA by RT-PCR

PCR products were separated in a 2.0% agarose gel and visualised by ethidium bromide staining (B) and probed with ^{32}P-labelled probe (A). RNA was isolated from alveolar macrophages (AM), monocyte/macrophages (M) from blood and lymph node (LN). The cDNA was amplified with ATPase primers. MW: 1-kb DNA ladder (GIBCO).

Fig.4.1



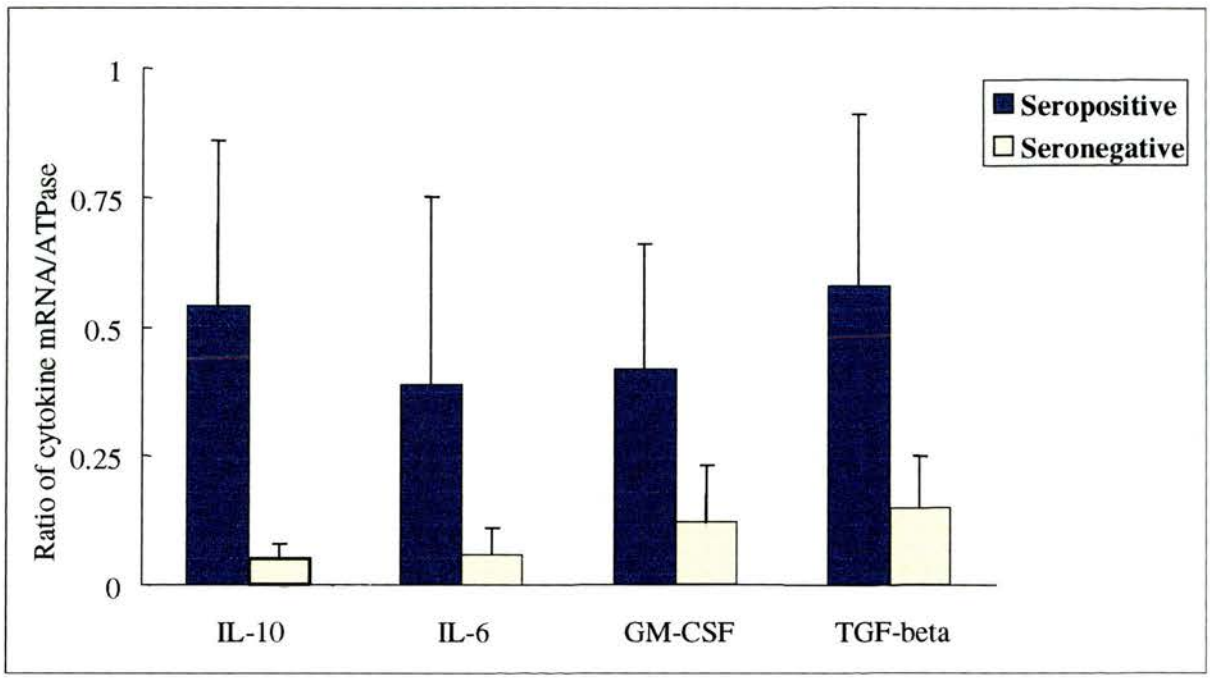


Figure 4.2 Cytokine mRNA level changes in AM in MVV infection

AM were isolated from naturally MVV-infected sheep (n=12) and control animals (n=3). Cytokine mRNA was determined by RT-PCR assay. IL-6 was amplified for 32 cycles, IL-10 for 30 cycles, TGF-β for 32 cycles, GM-CSF for 29 cycles. Results are expressed as the mean ± SD. Standard deviations are represented by error bars. There are significant differences between results with MVV-infected cells and those with uninfected cells ($P < 0.05$, Mann-Whitney nonparametric statistics).

Figure 4.3 The expression of mRNA for IL-6, IL-10, TGF-β and GM-CSF in blood Monocytes

Blood monocytes were isolated from naturally MVV-infected sheep and control animals and cytokines mRNA was determined by reverse RT-PCR assay and probed with ^{32}P-labelled probe. IL-6 was amplified for 32 cycles, IL-10 for 30 cycles, TGFβ for 32 cycles and GM-CSF for 29 cycles.

Lane 1: a seronegative animal.
Lanes 2, 3, 4: seropositive animals.

Figure 4.4 mRNA levels of IL-2, IL-6, IL-10, IFN-γ and GM-CSF from lymph nodes (LNs)

LNs were collected from naturally MVV-infected sheep and control animals. Cytokine mRNA was determined by RT-PCR assay (left) and Southern blot (right). IL-6 was amplified for 32cycles, IL-10 for 30 cycles, TGFβ for 32 cycles and GM-CSF for 29 cycles.

Lanes 1, 2, 3: seropositive animals:
Lane 4: a seronegative animal

Fig.4.3

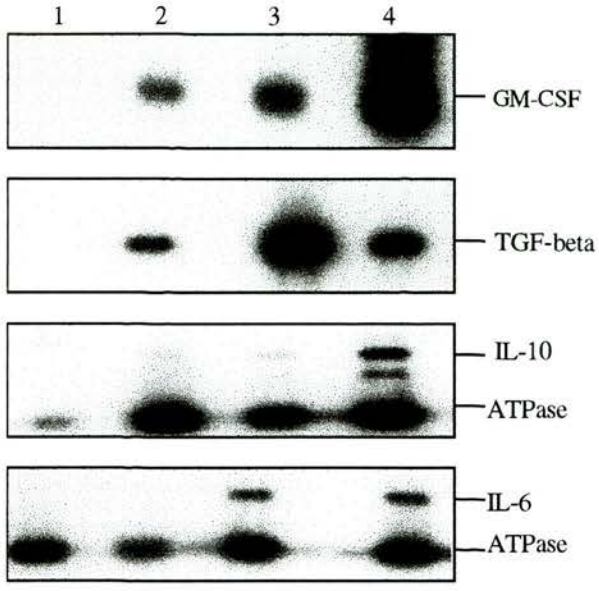
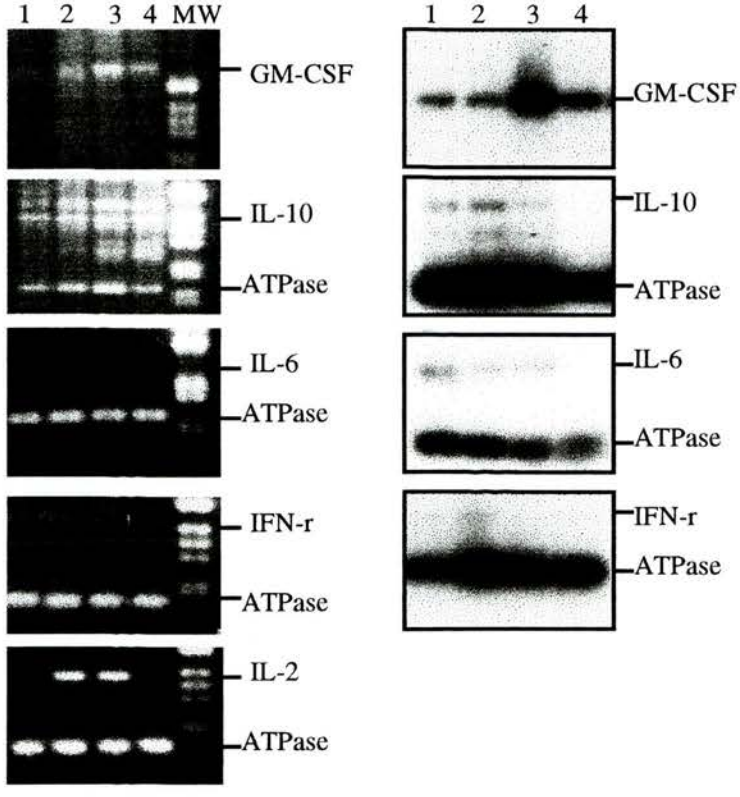

1
2
3
4
GM-CSF
TGF-beta
IL-10
ATPase
IL-6
ATPase

Fig.4.4


1 2 3 4 MW
GM-CSF
IL-10
ATPase
IL-6
ATPase
IFN-r
ATPase
IL-2
ATPase
1 2 3 4
GM-CSF
IL-10
ATPase
IL-6
ATPase
IFN-r
ATPase

The mRNA levels of GM-CSF showed no significant change in this small group. IFN-γ mRNAs were undetectable in both MVV-infected sheep groups and seronegative controls.

4.3.2.1: **Correlation of cytokine mRNA expression to the severity of the histological lung lesions**

The above results show that infection of AM with MVV results in alteration of cytokines. To determine whether cytokine mRNA level changed in relation to the severity of histological lesions in the lungs, a retrospective analysis of the quantity of cytokines mRNA in relation to the severity of the histological lung lesions was performed. Twelve MVV seropositive sheep were divided into two groups according to histological score in the lung (Table 4.2) and were tested.

Table 4.2 Pathological findings in sheep with and without lung lesions

	Sero status	Mean IR score	Mean SMH score	Mean FH score	Mean lesion score	Median Viral load*
Group A (n = 6)	+	2.4±0.34	1.8±0.41	27.5± 10.4	32.5±10.8	6.5x 10^5
Group B (n = 6)	+	0.5±0.09	0.20±0.05	0.00	0.5±0.07	0.6 x10^5

Group A = seropositive sheep with chronic lymphocytic interstitial pneumonia. Group B = seropositive sheep without chronic lymphocytic interstitial pneumonia. IR = interstitial reaction, SMH = smooth-muscle hyperplasia, FH = follicular hyperplasia. The severity of histological lesions in lung were measured by grading three parameters: IR, SMH, FH according to previously established criteria (Brodie *et al.*1992, 1995, Woodall *et al.* 1997). IR was defined by the thickening and cellular infiltration of interalveolar septae with lymphocytes (Woodall *et al.*1997). The degree of IR and SMH observed in sections from each animal was classified using the following scales: 0 = none, + = slight, ++ = moderate, and +++ = severe. A score was given to each category of lesion: 0 = 0, + = 1, ++ = 2, +++ = 3 and to each intermediate category: 0/+ = 0.5, +/++ = 1.5. ++/+++ = 2.5. The FH was evaluated by counting the number of lymphoid follicles in the sections and determining the number of follicles per cm^2 of tissue section. The mean surface studied for each animal was 10.2 cm^2. A more detailed description of this process is described elsewhere (Watt *et al.* 1992, Woodall *et al.* 1997). *Viral load is expressed as copy numbers per 500 ng cellular DNA

The mRNA levels of IL-6, IL-10, TNF-α, TGF-β and GM-CSF in AM of both groups are shown in Fig 4.5. The most striking differences between group A (with lung lesion) and group B (without lung lesions) were found for GM-CSF mRNA. In group A, high levels (0.52 ± 0.48) and group B low level (0.12 ± 0.39, $p< 0.05$) were determined (Fig.4.5). IL-10 and IL-6 mRNA expression were detected at similar levels in AM of both groups. Expression of TNF-α and TGF-β mRNA in AM showed no significant differences among both groups (Fig.4.5). The mRNA levels of IL-6, IL-10, TNF-α, TGF-β and GM-CSF in PBM of both groups showed no significant differences (Fig.4.5, $P<0.05$).

As demonstrated in Chapter 3, the level of MVV DNA and its replication status in AM from MVV infected sheep was correlated with histological lesions in the lung. However, the expression of IL-6, IL-10, TGF-β and TNF-α mRNA in AM showed no significant correlation to viral DNA load and viral RNA expression. The level of GM-CSF mRNA was correlated with the level of viral DNA. The association is particularly evident in some animals with high levels of GM-CSF mRNA and high levels of viral DNA load. In AM with the high level of GM-CSF mRNA (0.69 ± 0.39), MVV RNA was detectable. In contrast, MVV RNA was undetectable in AM with the low level of GM-CSF mRNA (0.19 ± 0.17) (Fig.4.6).

4.3.3: **The expression of cytokine genes by blood monocyte-derived macrophages infected with MVV *in vitro***

In order to clarify the influence of MVV in any possible regulation of the dysreguation of cytokine expression observed in AM of infected sheep, blood monocyte-derived macrophages were infected with MVV with low $TCID_{50}$ in order to mimic *in vivo* condition.

4.3.3.1: **MVV increases the expression of TGF-β, TNF-α and GM-CSF mRNA in macrophages *in vitro***

MDM were infected with MVV at 0.02 $TCID_{50}$ per cell. Mock infection with medium served as a control. After 2 h of incubation at 37°C, the cells were washed

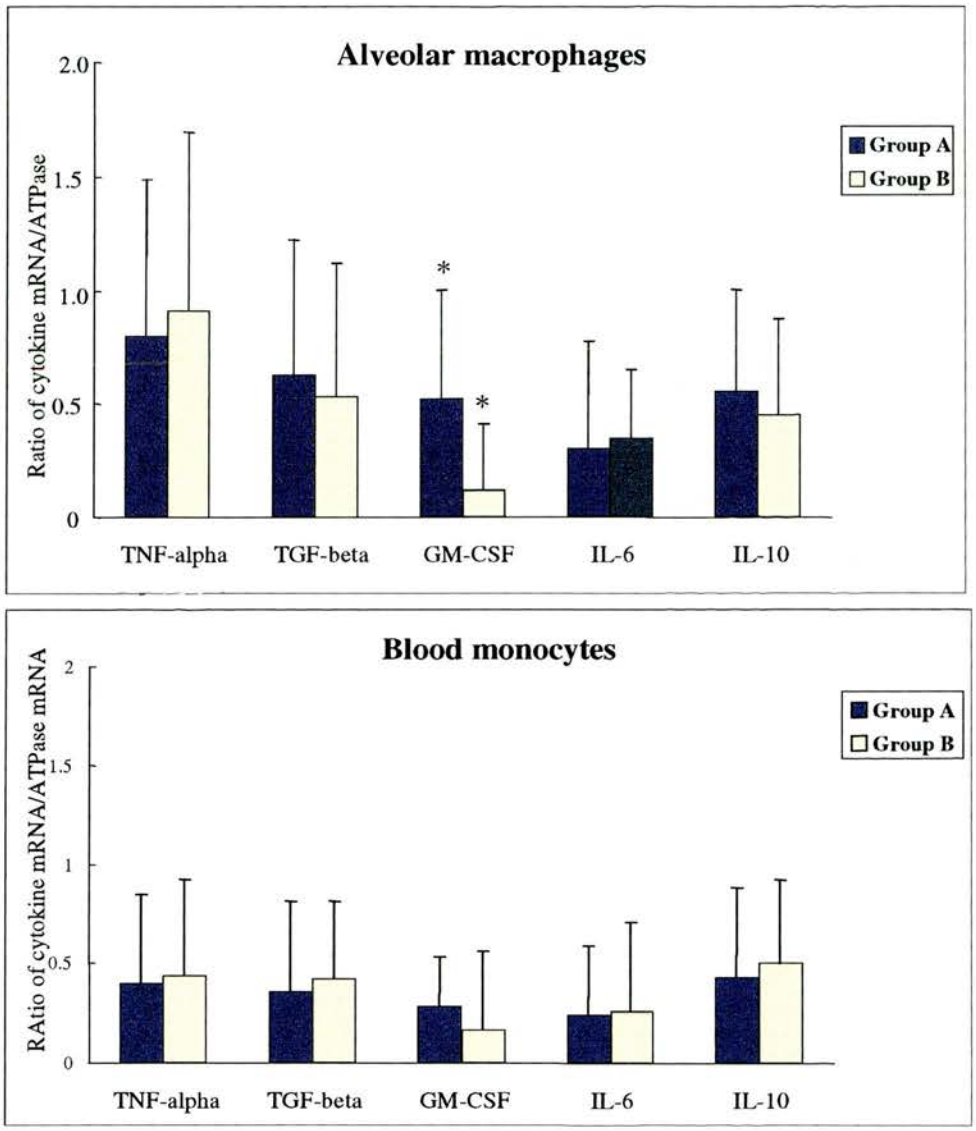


Figure 4.5 Comparison of macrophage cytokine mRNA in sheep with and without lung lesions

Relative level of cytokine mRNA was determined by RT-PCR as described in Material and Methods. Results were presented as the ratio of the signal from each cytokine mRNA to the signal obtained from ATPase mRNA. Bars represent the mean relative level of cytokine mRNA/ATPase mRNA and error bars are the standard deviation. Group A: animals with lung lesions (n=6). Group B: animals without lung lesions (n=4). Results are expressed as the mean ± SD. Standard deviations (SD) are represented by error bars. Statistically significant differences ($P < 0.05$, Mann-Whitney nonparametric statistics) between results with group A and those with group B are denoted by asterisks.

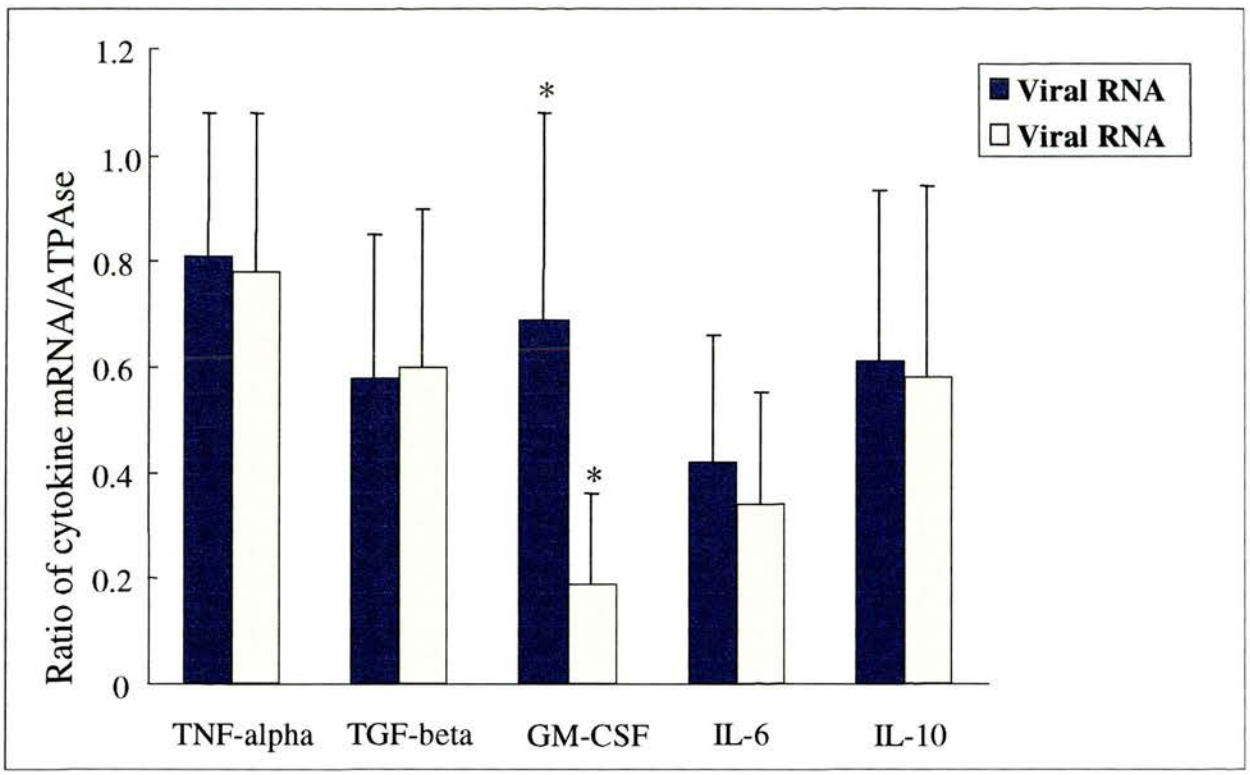


Figure 4.6 Cytokine mRNA levels in AM from infected sheep with or without detectable viral RNA

AM from infected sheep with or without detectable viral RNA were subjected to RT-PCR amplification for detection of cytokine mRNA. PCR products for cytokines were separated on 2% agarose gel and analysed by autoradiography and densitometry. The results are shown as the ratio between cytokine mRNA/ATPase mRNA. Results are expressed as the mean ± SD. Standard deviations (SD) are represented by error bars. Statistically significant differences ($P < 0.05$, Mann-Whitney nonparametric statistics) between results with viral RNA (+) group and those with viral RNA (-) group are denoted by asterisks.

RNA(+): MVV RNA is detectable in AM(n=3).

RNA(-): MVV RNA is undetectable in AM(n=3).

to remove the virus inoculum and total RNA was isolated from these cultures at the indicated time post-infection. Cells infected with MVV for 4 hrs showed an increase in the expression of the mRNA for TNF-α and TGF-β compared with that of uninfected controls (Fig.4.7 and 4.8). To estimate the relative expression of both cytokines, a semi-quantitative RT-PCR was used and amplification of cytokine cDNA was standardised to the housekeeping gene ATPase. As shown in Fig 4.7, the mean expression of mRNA for TNF-α and TGF-β in MVV infected macrophages was significantly higher than that of uninfected controls ($P < 0.05$, Student's t test). This significant increase was not seen when the cultures were treated with heat-inactivated MVV. Levels of TGF-β and TNF-α mRNA in these MDM were similar relative to uninfected MDMs but markedly lower than in live MVV-infected MDMs (Fig 4.7), which suggests that viral replication is required for increased expression of mRNA for TNF-α and TGF-β in macrophages. In contrast, although increased levels of GM-CSF mRNA were observed in MVV-infected MDMs, these were not significantly different from the uninfected MDMs ($P>0.05$, Student's t test). IL-10 and IL-6 mRNA level remained unchanged (Fig.4.7).

To follow the expression of TNF-α mRNA, total RNA was extracted from MDM lysates collected at different times after infection, the level of the cytokine expression were detected by relative RT-PCR. The significant expression of TNF-α mRNA level was detected at 6 hours after infection and its level increased with the time of postinfection (Fig.4.9 and 4.10, $P < 0.05$, Mann-Whitney nonparametric statistics) although the increased level of TNF-α mRNA was detected at 2 hours after infection (Fig. 4.10, $P > 0.05$, Mann-Whitney nonparametric statistics).

4.3.3.2: **Infection of macrophages with MVV failed to activate expression of IL-10 and IL-6 mRNA *in vitro***

The effects on IL-10 expression were investigated during the MVV infection and viral production. IL-10 mRNA was quantified by using RT-PCR during the first 24 hours after MDM infection with MVV $TCID_{50}$ ranging from 0.02 to 2 per cell. The infection was verified 24 hours after infection by DNA PCR. At no point before or

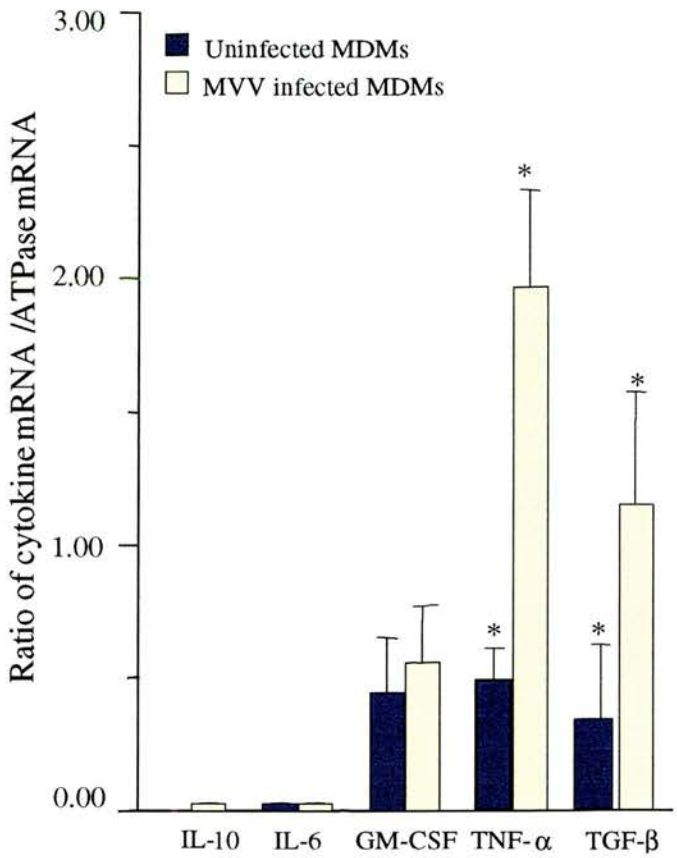


Figure 4.7 Levels of various cytokines detected in cultures of MDMs infected with MVV

MDMs were infected with MVV at 0.02 $TCID_{50}$ per cell. Mock infection with medium served as a control. After 2 h of incubation at 37°C, the cell were washed to remove the virus inoculum and total RNA was isolated from these cultures at 4hrs postinfection. The expression of mRNA for IL-6, IL-10, TNF-α, TGF-β and GM-CSF was determined by RT-PCR assay and analysed by autoradiography and densitometry. IL-6 was amplified for 32cycles, IL-10 for 30 cycles, TNF-α for 30 cycles, TGF-β for 32 cycles, GM-CSF for 29 cycles and ATPase for 35 cycles. The results are shown as the ratio between cytokine mRNA/ATPase mRNA. Results are expressed as the mean ± SD. Standard deviations (SD) are represented by error bars. Statistically significant differences ($P < 0.05$, Student's t test) between results with MVV-infected cells and those with uninfected cells are denoted by asterisks.

Figure 4.8 MVV increases the expression of TGF-β and TNF-α mRNA in macrophages

MDMs were infected with MVV at 0.02 $TCID_{50}$ per cell. Mock infection with heat-inactivated MVV served as a control. After 2 h of incubation at 37°C, the cell were washed to remove the virus inoculum and total RNA was isolated from these cultures at 4 hrs postinfection. The expression of cytokine mRNA in MDMs infected with live MVV or heat-inactivated MVV was determined by RT-PCR and Southern blot analysis as described in Material and Methods of Chapter 4. P: positive control. N: negative control.

A: The expression of mRNA for TGF-β in MDMs.
Lane 1: uninfected.
Lanes 2, 3: heat-inactivated MVV,
Lanes 4, 5, 6: MVV

B: The expression of mRNA for TNF-α in MDMs.
Lanes 1, 2, 3: MVV-infected MDMs.
Lanes 4, 5: uninfected MDMs.

Figure 4.9 Kinetics of expression of TNF-α mRNA in MVV infected MDMs

MDMs were cultured at a concentration of $5x10^5$ per well and infected with MVV at 0.02 $TCID_{50}$ per cell. Cells were harvested at the indicated time after infection. TNF-α and expression were detected by by RT-PCR. 27 cycles for TNF-α were carried out.

Fig.4.8

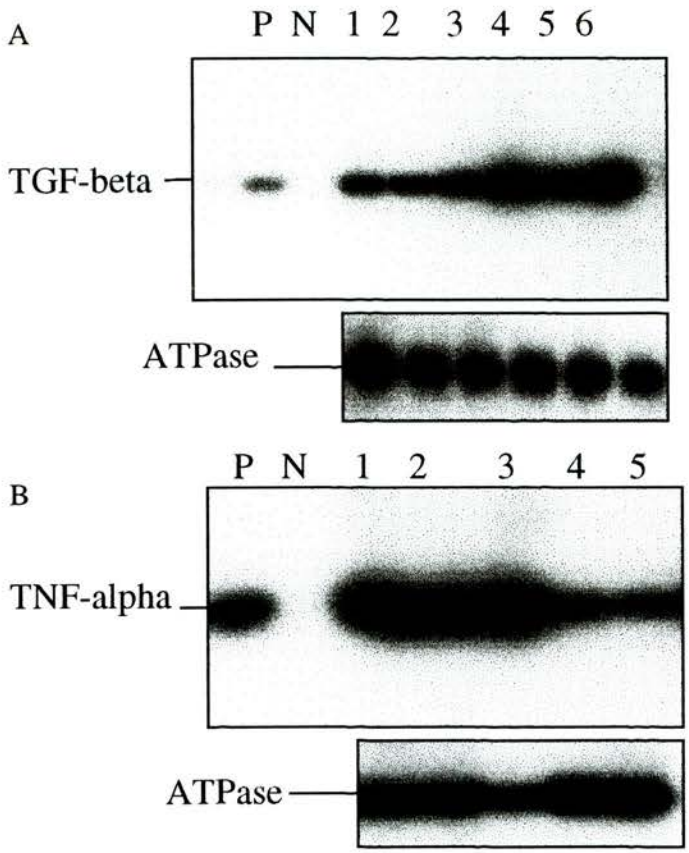
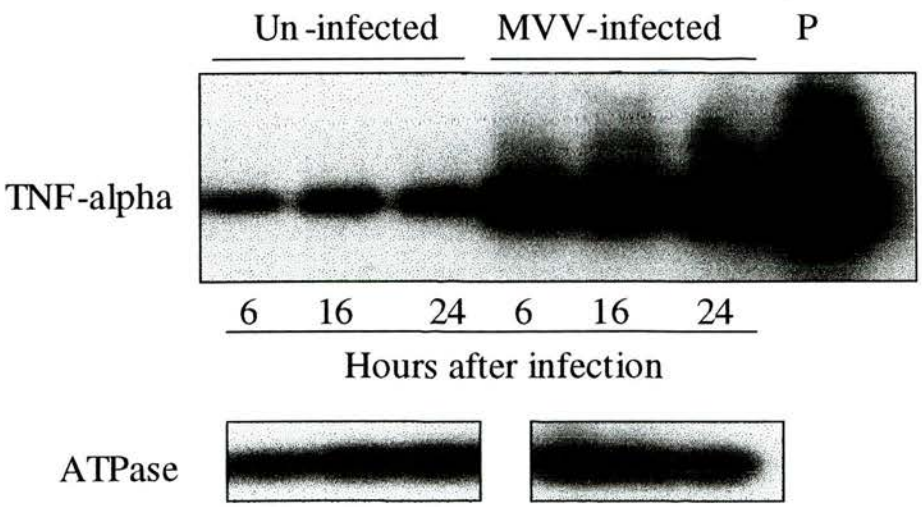

A
P N 1 2 3 4 5 6
TGF-beta
ATPase
B
P N 1 2 3 4 5
TNF-alpha
ATPase

Fig.4.9


Un-infected
MVV-infected
P
TNF-alpha
6 16 24 6 16 24
Hours after infection
ATPase

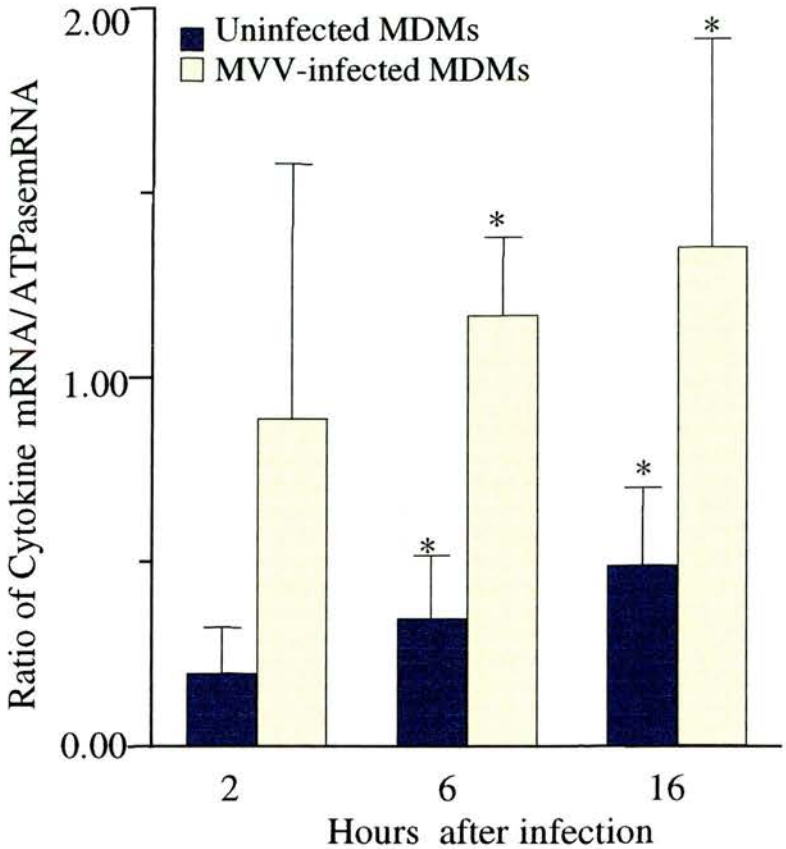


Figure 4.10 Kinetics of expression of TNF-α mRNA in MDMs

MDMs were infected with MVV at 0.02 $TCID_{50}$ per cell. Mock infection with medium served as a control. After 2 h of incubation at 37°C, the cells were washed to remove the virus inoculum and total RNA was isolated from these cultures at the indicated time post-infection. The level of TNF-α mRNA was determined by RT-PCR. 27 cycles for TNF-α were carried out. Each point is presented as mean ± SD of three samples. Results are expressed as the mean ± SD. Standard deviations (SD) are represented by error bars. Statistically significant differences ($P < 0.05$, Mann-Whitney non-parametric statistics) between results with MVV-infected cells and those with uninfected cells are denoted by asterisks.

after infection could IL-10 mRNA be detected in MDM although the expression of TNF-α mRNA was seen (Fig.4.11).

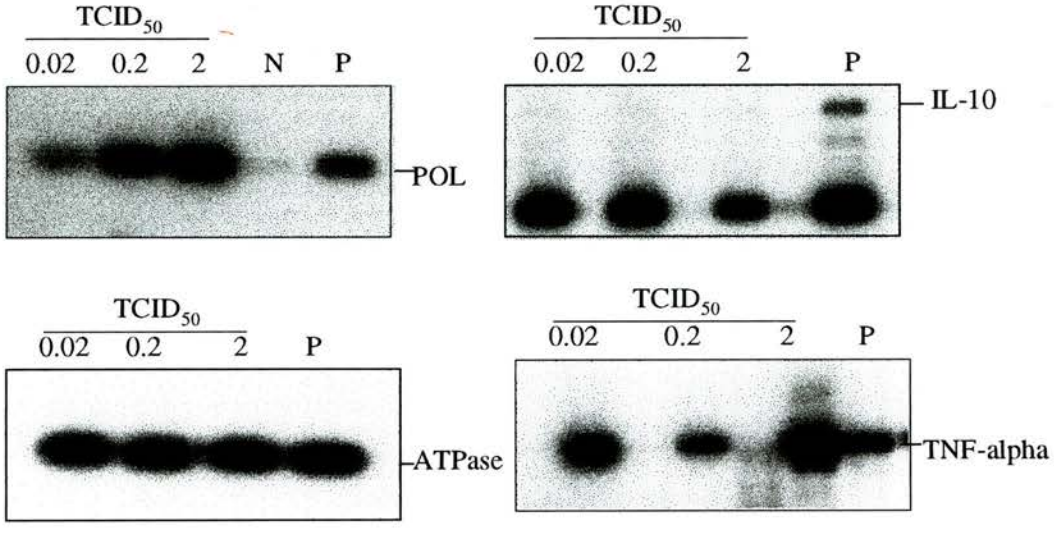
MVV infection did not induce increased expression of IL-10 mRNA in infected MDMs at the time points up to 24 hours. However, it remained possible that MVV infection might induce IL-10 during productive infection. To explore this possibility, The expression of IL-10 mRNA was investigated in MDM 3 days after infection tested. No increased expression of IL-10 was detected up to 3 days after infection (Fig.4.12). In lipoplysaccharide (LPS)-stimulated MDM, IL-10 mRNA was detected (Fig.4.12). Therefore, these findings suggest that MVV infection of MDMs is not sufficient to induce detectable level of IL-10 in these cells. Neither early virus-cell contact with low nor high $TCID_{50}$ induced the expression of IL-10 mRNA in MDMs. In MVV infected sheep, overexpression of TNF-α in lung was reported (Woodall *et al.* 1997). TNF-α has been described as inducing IL-10 expression (Wanidworanun *et al.* 1993). However, under our culture conditions, no IL-10 mRNA was observed in uninfected and MVV-infected MDMs whereas TNF-α mRNA were observed in MVV infected MDM (Fig.4.11).

The above results have shown that infection of macrophages with MVV failed to activate expression of IL-10 mRNA. In view of the potential influence of the state of maturation of monocytes into macrophages in response to lentivirus infection (Kazaz1 *et al.* 1992), this result would not exclude that the induction of IL-10 expression by MVV is linked to cell differentiation. To test this possibility, monocytes were infected with MVV at 0.02 $TCID_{50}$ per cell ether on the day of isolation of cells (D.0) or cells cultured for 7 days (D.7). As a control, the same virus stock was used the same day to stimulate both cells. When compared with uninfected cells, IL-10 mRNA was detected in monocytes on the day of isolation (Fig.5.13). In contrast, in macrophages cultured for 7 days, no increased expression of IL-10 mRNA was detected in response to MVV infection compared to uninfected cells (Fig.4.13). Because the amount of expression of cytokine mRNA was

Figure 4.11 Expression of viral DNA, IL-10 and TNF-α mRNA in MDMs

MDMs were infected with three different $TCID_{50}$/cell (0.02, 0.2, 2) of MVV EV1. Mock infection with medium served as a control. After 2 h of incubation at 37°C, the cells were washed to remove the virus inoculum and total RNA for cytokines and DNA for virus was isolated from these cultures at 24 hrs postinfection. The expression of mRNA for IL-10 was determined by RT-PCR assay and analysed by autoradiography. Viral DNA was detected by PCR using a pair of primers specific for MVV *pol* gene.

Fig.4.11


TCID50
0.02
0.2
2
N
P
POL
TCID50
0.02
0.2
2
P
IL-10
TCID50
0.02
0.2
2
P
ATPase
TCID50
0.02
0.2
2
P
TNF-alpha

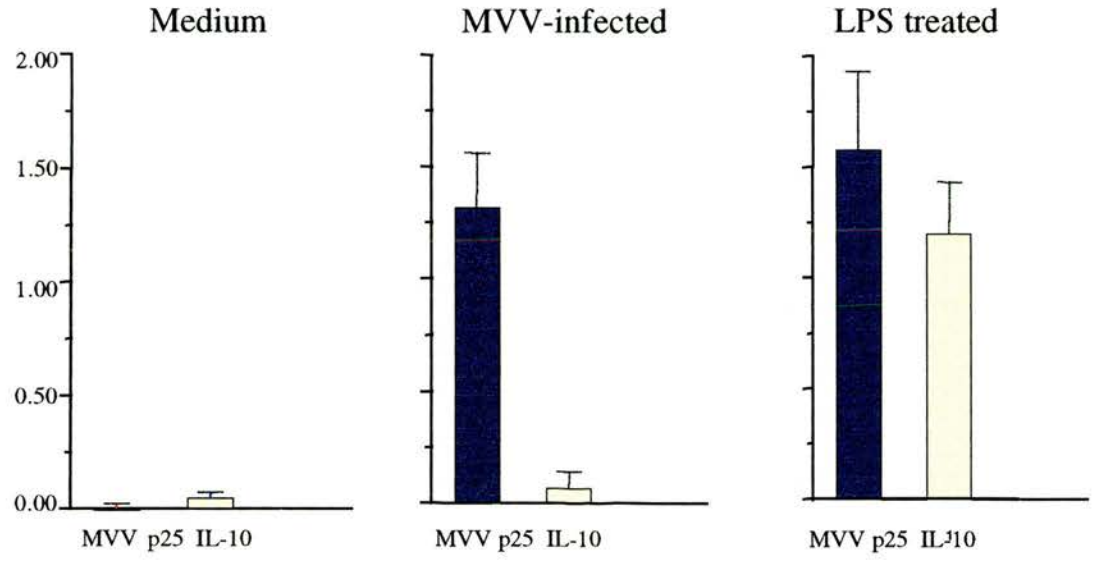


Figure 4.12 Cellular viral Load and IL-10 mRNA expression in MDMs

MDMs were infected with 0.02$TCID_{50}$/cell of MVV EV1. At 3 day postinfection total RNA was isolated from these cultures and culture supernatants were collected for detection of viral p25. The expression of mRNA for IL-10 was determined by RT-PCR assay and analysed by autoradiography. Viral P25 was detected by competitive ELISA as described in Chapter 2. The level of IL-10 mRNA is expressed as the mean of ratio of IL-10 mRNA to ATPase mRNA. Viral p25 level is expressed as ng/ml x 10.

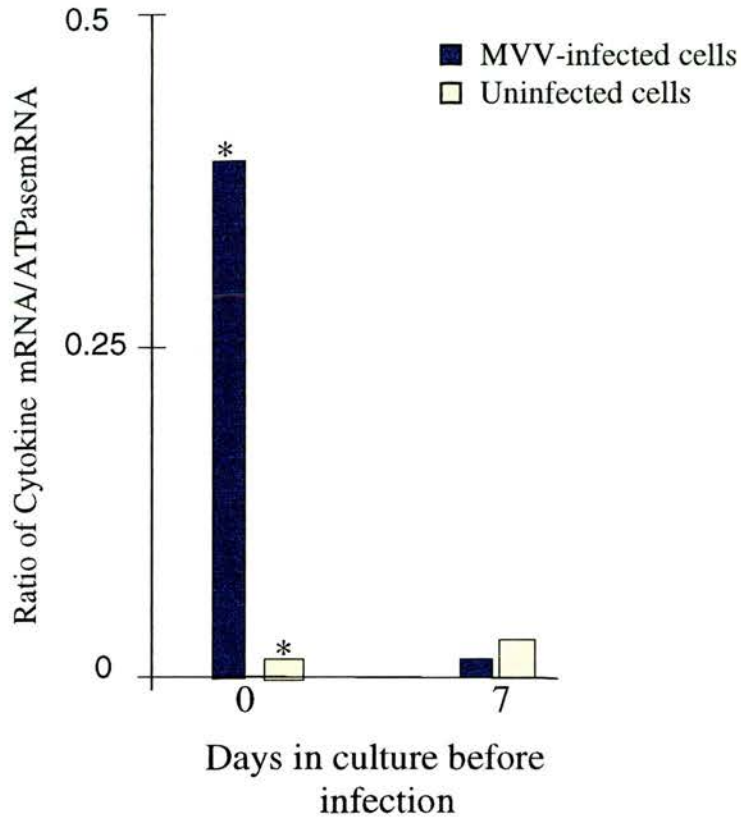


Figure 4.13 Induction of expression of IL-10 mRNA by MVV infection during cell culture of monocytes

Monocytes were cultured in 24-well plates. At the indicated times, cells were exposed to MVV at a 0.02 $TCID_{50}$ per cell or control medium. After 6 hours, expression of cytokines mRNA was determined by relative RT-PCR. These results are representative of three other experiments. The data is expressed as the mean of ratio of IL-10 mRNA to ATPase mRNA. Statistically significant differences ($P < 0.05$, Mann-Whitney non-parametric statistics) between results with MVV-infected cells and those with uninfected cells are denoted by asterisks.

proportional to the numbers of monocytes, macrophages were harvested with treatment of EDTA/PBS after 7 days of culture and re-cultured at the higher cell density. They still did not express detectable cytokine mRNA when challenged with MVV. This excludes the possibility that the lack of IL-10 expression in response to MVV was not caused by an infection-related-loss of macrophage viability. Stimulation with LPS led to induction of IL-10 mRNA in both freshly isolated monocytes and macrophages cultured for 7 days indicating that the loss of ability to express IL-10 was selective as monocytes differentiate into macrophages (data not shown). As wtih IL-10, no expression of IL-6 mRNA could be observed in MVV-infected MDM when compared with those uninfected MDM (medium control) (Fig.4.14 and 4.15). In certain experiments, the cultures were extended for up to 4 days after infection. Even at these late time points, no significant expression of IL-6 mRNA was found in MVV-infected MDMs.

4.3.3.3: **The effect of exogenous cytokine on the expression of cytokine gene in macrophages**

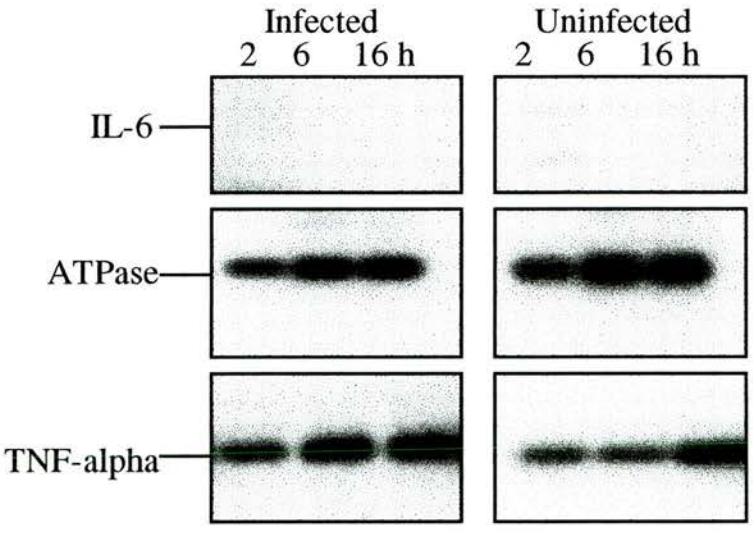
As demonstrated in the above results, lack of IL-10 and IL-6 mRNA expression was observed in MVV-infected MDM and the increased level of GM-CSF mRNA was found in AM of MVV infected sheep. It is possible that MVV infection might induce one cytokine expression which in turn induces the expression of another cytokine. To explore this possibility, the effects of GM-CSF on the expression of IL-6 mRNA in MDMs was investigated. As shown in Fig.4.16, when MVV infected-MDMs are stimulated with GM-CSF the expression of IL-6 mRNA was observed within 24 hours. However, increased expression of IL-6 was detected in MDMs exposed to GM-CSF and GM-CSF+MVV but not in MVV alone, suggesting that interaction of MVV with the target cells is not essential for the expression of IL-6.

Similarly, it was found that exogenous IFN-γ was able to modulate the TGF-β mRNA expression. As shown in Fig.4.17, Levels of TGF-β mRNA in MDMs treated with heat-inactivated IFN-γ were similar relative to uninfected MDMs but markedly lower than in live MVV-infected MDMs.

Figure 4.14 Lack of the detectable expression of IL-6 mRNA

MDMs were cultured at a concentration of $5x10^5$ per well and infected with MVV at 0.02 $TCID_{50}$ per cell. Cells were harvested at the indicated time after infection. TNF-α and IL-6 mRNA expression were detected by RT-PCR.

Fig.4.14



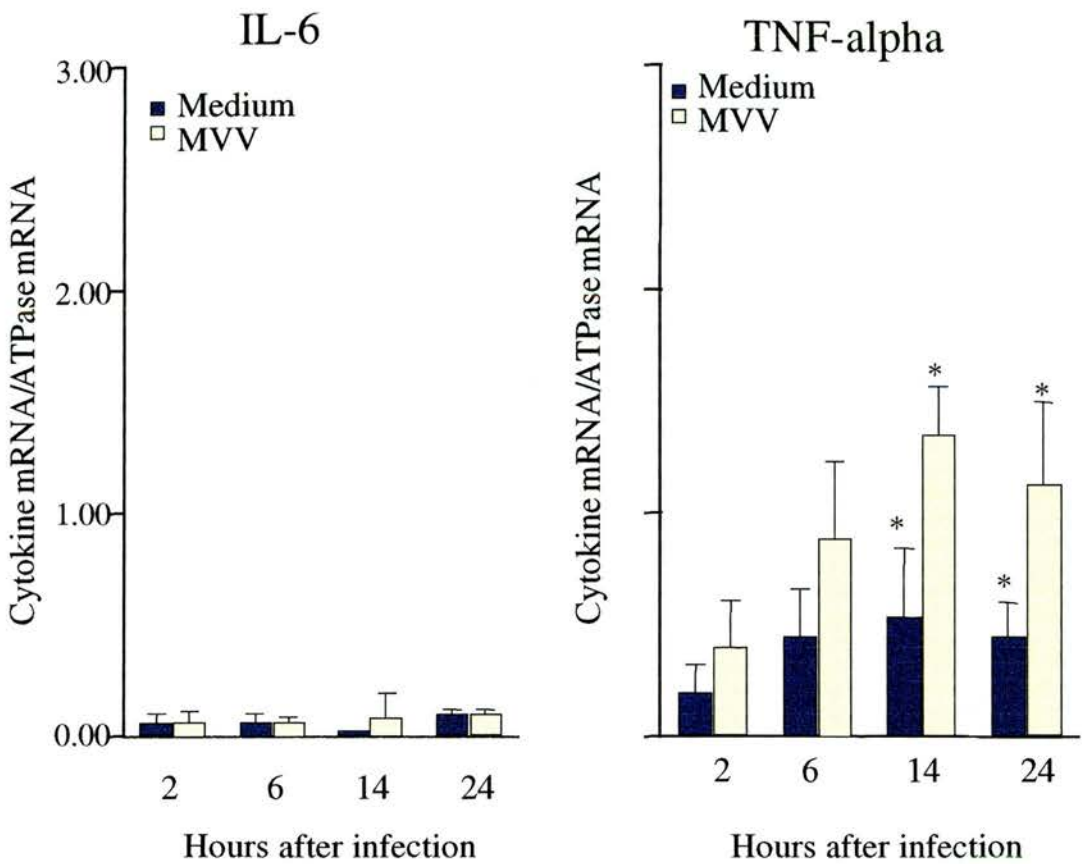


Figure 4.15 Lack of IL-6 mRNA detectable in MDMs infected by MVV *in vitro*

MDMs were infected with 0.02$TCID_{50}$/cell of MVV EV1. Medium served as a control. After 2 h of incubation at 37°C, the cells were washed to remove the virus inoculum. Total RNA and DNA were isolated from these cultures at 2, 6, 12, 24 hours post-infection, and culture supernatants were collected for detection of viral p25. The expression of mRNA for IL-6 was determined by RT-PCR assay and analysed by autoradiography. Viral DNA was detected by QC-PCR as described in Chapter 3. The level of IL-6 mRNA is expressed as the mean of ratio of IL-6 mRNA to ATPase mRNA. Results shown are the mean (± SD) of three separate experiments. Standard deviations (SD) are represented by error bars. Statistically significant differences ($P < 0.05$, Mann-Whitney non-parametric statistics) between results with MVV infected cells and those with uninfected cells are denoted by asterisks.

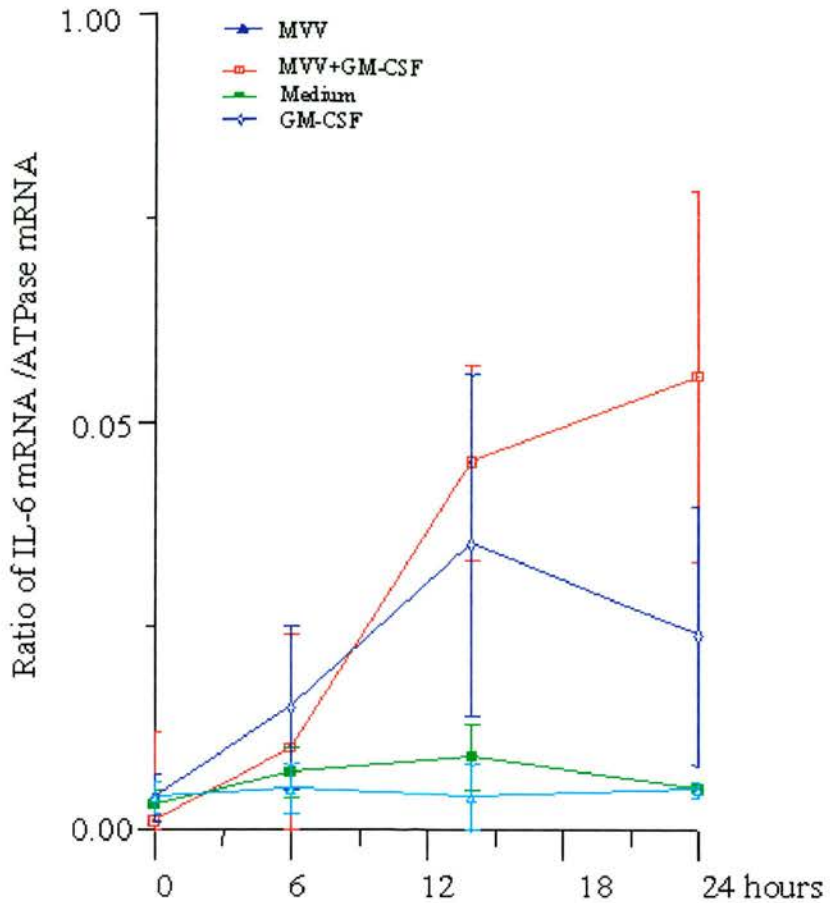


Figure 4.16 Effects of GM-CSF on IL-6 mRNA expression by MVV-infected macrophages

MDMs were exposed to GM-CSF (10 ng/ml) and challenged with 0.02$TCID_{50}$/cell of MVV EV1. Cells treated with GM-CSF alone, medium alone and MVV alone were served as controls. After 2 h of incubation at 37°C, the cells were washed to remove the virus inoculum. Total RNA was isolated from these cultures at 6, 12, 18, 24 hours post-infection. The expression of mRNA for IL-6 was determined by RT-PCR assay and analysed by autoradiography. The level of IL-6 mRNA is expressed as the mean of ratio of IL-6 mRNA to ATPase mRNA. Each point represented mean + SD of three experiments.

Figure 4.17 Comparison of TGF-β mRNA expression in MDMs treated with IFN-γ or heat-inactivated IFN-γ

PCR products were separated in a 2.0% agarose gel and analysed by autoradiography and densitometry.

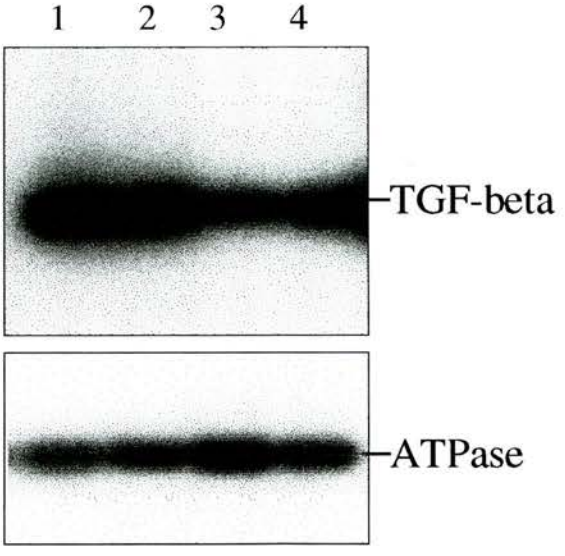
Lane 1: IFN-γ (10ng/ml)+MVV.

Lane 2: MVV.

Lane 3: Inactivated IFN-γ (10ng/ml)+uninfected

Lane 4: uninfected.

Fig.4.17



CHAPTER 4:

Discussion

The levels of expression of IL-10, IL-6, TGF-β, TNF-α and GM-CSF mRNA expression were semi-quantified by measuring the level of mRNA using semi-quantaitve RT-PCR in AM, blood monocytes after infection *in vivo* and *in vitro.*

The increased expression of GM-CSF mRNA was observed in AM of MVV infected sheep. However, *in vitro* studies with MDM at low of $TCID_{50}$ showed that no significant increase in the GM-CSF mRNA expression of MDM was found after infection. It is possible that this expression may be restricted to infected cells because MDM were infected with MVV at low $TCID_{50}$ and harvested at the time that only a proportion of the cells were infected. A high level of GM-CSF was expressed in AM of MVV infected sheep with pathological lung lesions (group A) where the viral RNA is detectable. This result, together with the previous study that the number of infected AM increased with the severity of lung lesions (Brodie *et al* 1992), indicate that the level of GM-CSF expression is related to viral replication.

This response of elevated expression of GM-CSF may, in part, responsible for driving active replication of the virus in AM in the later stage of disease. It is well known that GM-CSF up-regulates antigen-presenting activity of dentritic cells and recruits these cell to localised inflamed regions of the lung, thus promoting lymphoid follicles development. GM-CSF also mediates abrogation of suppression of T lymphocyte activities in the lung by AM, causing T lymphocytes accumulation to occur. Thus it is possible that increased level of GM-CSF in the lung may account for development of lymphoid follicles in the lung. It is tempting, therefore, to hypothize that MVV infection may cause progression of disease via a profound perturbation of the cytokine network. A recent study of TGF-β expression in ovine lentivirus-induced lymphoid interstitial pneumonia showed the increased level of TGF-β expression in lymphoid follicles (Moreno *et al* 1998), providing further evidence of a direct pathogenic consequence of MVV infection via a profound

perturbation of the cytokine network

The levels of TNF-α and TGF-β mRNA was elevated in macrophages infected with MVV *in vivo* and *in vitro*. TGF-β has been shown to be a potent chemoattractant for monocytes and macrophages. Additionally, TGF-β is known to induce monocyte secretion of cytokines and may be important in augmenting and perpetuating inflammatory reactions (Wahl *et al.* 1990). Increased activity of TGF-β in the affected sites may therefore result in enhanced chemoattractant activity for monocytes and macrophages and augmentation and perpetuation of inflammatory reactions via cytokine release. In exploring whether MVV itself could be a trigger for the enhanced expression of TGF-β and TNF-α, a significant increase in TGF-β and TNF-α was not seen when the cultures were treated with heat-inactivated MVV. In these cells, levels of TGF-β and TNF-α were similar to uninfected MDMs but markedly lower than in live MVV-infected MDMs (Fig 4.8), which suggests that viral replication is required for increased expression of mRNA for TNF-α and TGF-β in macrophages. However, it is unclear whether this expression is restricted to infected MDM or even if other uninfected cells have contributed.

The increased expression of IL-6 and IL-10 mRNA was observed in AM from infected sheep. However, *in vitro* study showed no detectable expression of IL-10 mRNA and IL-6 mRNA in MDMs in response to MVV infection, indicating that other factors may be involved in contributing to IL-6 or IL-10 expression in macrophages in response to MVV *in vivo*. In HIV infection, IL-6 expression is generally not elevated in patients during their primary infection with HIV, but then increases toward the end of the acute phase when viral burden is decreasing (Sinicco *et al.* 1993), suggesting that the increase in IL-6 expression is not related with the virus replication. The results of the study that IL-10 mRNA was detected in freshly isolated monocytesbut not in cultured macrophages in response to MVV infection, suggest that the failure to induce IL-10 expression by MVV may be linked to cell differentiation. Furthermore, GM-CSF was found to stimulate the expression of IL-6 mRNA in MDMs, suggesting the possible mechanism that MVV might induce

expression of IL-6 *in vivo* by an indirect process, i.e., MVV may induces the expression of GM-CSF. This may in turn trigger the expression of IL-6 mRNA in AM. Thus the interaction among cytokine have, in part, a role in regulation of cytokine expression in macrophages during viral infection (Foli *et al.* 1997).

The factors responsible for initiating a switch from asymptomatic to symptomatic disease expression in lentiviral infections are not yet clear. Th1 and Th2 response in mice play a critical role in viral clearance (Braciale and Braciale 1994). The Th2 cytokine response has been suggested to response for disease progression during HIV infection (Clerici and Shearer, 1993). Patients with Th1-type response, characterised by production of IL-2 and IFN-γ remain as asymptomatic whereas those that switch from Th1 to Th2, as indicated by decreased secretion of IL-12 and increased production of IL-4, develop clinical diseases (Clerici and Shearer 1993, Chehimi *et al.* 1994). In FIV-infected cat asymptomatic cats produces high level of IL-2 (a Th1 cytokine) and high level of Il-6 (a Th2 cytokine) (Lawrence *et al.* 1995). In this study, both Th1 and Th2 cytokine expression was seen in LNs. Similarly, a study of cytokine expression in CAEV-induced arthritis (CAEV is closely related to MVV) extended these observations showing both Th1 and Th2 cytokine expression in the same arthritis joints but in different regions (Lechner *et al.* 1997). On the basis of this finding, it is hypothesed that both types of cytokines could be expressed concurrently but in different regions of MVV-infected tissue. However, studies monitoring changes in levels of IL-10, IL-4 and IFN-γ during MVV infection are required before a definite role of a shift from Th1 to Th2 in the immunopathology of MVV infection can be determined. Whether or not a Th1/Th2 switch occurs, it is obvious from these observations that interaction between lentiviruses and host cells invariably results in modulation of cytokine expression and a possible dysregulated pattern of cytokine expression in response to the exogenous stimulus.

CHAPTER 5

EFFECT OF THE CYTOKINE GM-CSF, TGF-β AND IFN-γ ON MAEDI-VISNA VIRUS REPLICATION

5.1: **Introduction**

MVV is genetically and pathogenically similar to HIV. A number of studies have demonstrated that HIV replication in monocytic cells is particularly sensitive to modulation by cytokines (see Chapter 1). IL-1, IL-3, IL-6, TNF-α, TNF-β, IFN-γ, and GM-CSF have been shown to be capable of up-regulating HIV in these mononuclear phagocytes (Poli *et al.* 1993, Bornmann *et al.* 1997). Other cytokines such as IL-4 and IL-10 on the other hand, have generally been reported to suppress HIV replication (Poli *et al.* 1990, Saville *et al.* 1994, Weissman *et al.* 1994). Furthermore, the results of the studies demonstrated the level of GM-CSF mRNA expression was correlated with the viral DNA load, indicating that cytokines might play an important role in regulating MVV replication or that virus replication plays a role in cytokine expression.

Despite a large number of reports about the role of cytokines in regulating HIV replication, there are relatively little data concerning the role of cytokines in modulating MVV replication. Ellis *et al.* (1994) showed that TNF-α and IL-1β enhance on MVV expression in AMs *in vitro*. In addition, it has been demonstrated that bovine recombinant IFN-α has an inhibitory effect on MVV in AM (Ellis *et al.* 1994). Indeed, like other lentiviruses, it has become apparent that there is a complex pattern of interaction between the regulation of cytokines and virus replication and that such interactions may have a critical role in induction of MVV expression and in the pathogenesis of maedi-visna disease. Thus the elucidation of the effect of cytokines on MVV expression is a crucial step in the determining immuno-pathological mechanisms involved in lentiviral disease progression. To address this question, the effects of recombinant GM-CSF, IFN-γ and TGF-β on MVV replication in monocyte-derived macrophages (MDMs) were investigated.

CHAPTER 5:

Materials and methods

5.2.1: **Reagents**

Recombinant ovine GM-CSF (0.65 mg/ml) was kindly provided by Dr DM Haig Moredun Research Institute, Scotland. Recombinant bovine interferon gamma (IFN-γ, 1mg/ml, $2.5x10^6$ u/mg) was obtained from Ciba Geigy S.A. (Basel, Switzerland). This has been previously demonstrated to be active on ovine AMs (Nash *et al.* 1992, Ellis *et al.* 1994). Human recombinant TGF-β was obtained from Genosys (UK).

5.2.2: **Preparation of viral stocks**

MVV EV1 was propagated as described in Chapter 2. Virus containing supernatant was then filtered though a 0.4 μm filter to remove cell debris, treated with DNase at 37°C for 60 minutes to remove viral DNA and stored at -70°C.

5.2.3: **Cytokine treatment and MVV infection of MDMs**

Monocyte-derived macrophages (MDM) was isolated and cultured as described in Chapter 2. Cytokines were added to 5 to 7 day-old macrophage cultures 1 day before infection. Treated and untreated macrophages were inoculated with cell-free, DNase treated MVV culture at 0.02 $TCID_{50}$ per cell and allowed to absorb for 2 hours before complete aspiration of medium, washing and addition of fresh medium. Medium containing cytokine was replenished every 3 days. The supernatants were stored for quantification of MVV p25.

CHAPTER 5:

Results

5.3.1: **Morphology of blood monocytes and MDMs**

Figure 5.1 shows the morphology of monocytes and MDMs. During differentiation, monocytes underwent a marked increase in cell size with a concomitant increase of the nucleus-cytoplasmic ratio (Fig5.1). The nucleus of the monocytes rounded off after the second day and was located centrally or slightly eccentrically within the cells. On day 7, the cytoplasm was characterised by numerous vacuoles. Macrophages, after 7 days culture, exhibited an extremely irregular surface and numerous pseudopodia on cell membrane (Fig.5.1B). Cell populations exhibited a certain morphological heterogeneity. This phenomenon may reflect the presence of different maturation stages of the cells or early acquisition of characteristics of tissue macrophages by sub-populations of the cultured cells.

5.3.2: **Effect of maturation/differentiation of monocytes on MVV replication in these cells**

In view of the potential influence of the state of maturation or duration of adherence of monocytes on MVV replication, a series of experiments were designed to test this hypothesis.

5.3.2.1: **Reverse transcription in MVV-infected fresh and cultured monocytes**

To study the influence of cellular differentiation on the susceptibility of monocytes /macrophages to MVV infection and virus replication, monocytes were inoculated with EV1 at 0.02 $TCID_{50}$/cell on the day of isolation and day 7 in culture, and then lysed at 12h, 48h and 7 days after infection. The cell lysates were analysed by PCR with primers to MVV *pol*. The *pol* DNA were detectable within infected MDM after 7 days culture by 12 hours, with the amount of the *pol* DNA increasing with the time of infection (Fig5.2). In contrast, freshly isolated monocytes exposed to MVV generated no detectable viral DNA, even up to 7 days. later. This result supports the

Figure 5.1 Morphology of monocytes and monocytes-derived macrophages (MDMs)

Monocytes were isolated from peripheral blood mononuclear cells by means of plastic adherence and cultured in medium containing 10% FCS.

A: monocytes on the day of isolation (light microscope, original magnification x 200).

B: macrophages after 7 day culture (light microscope, original magnification x 200).

Fig.5.1

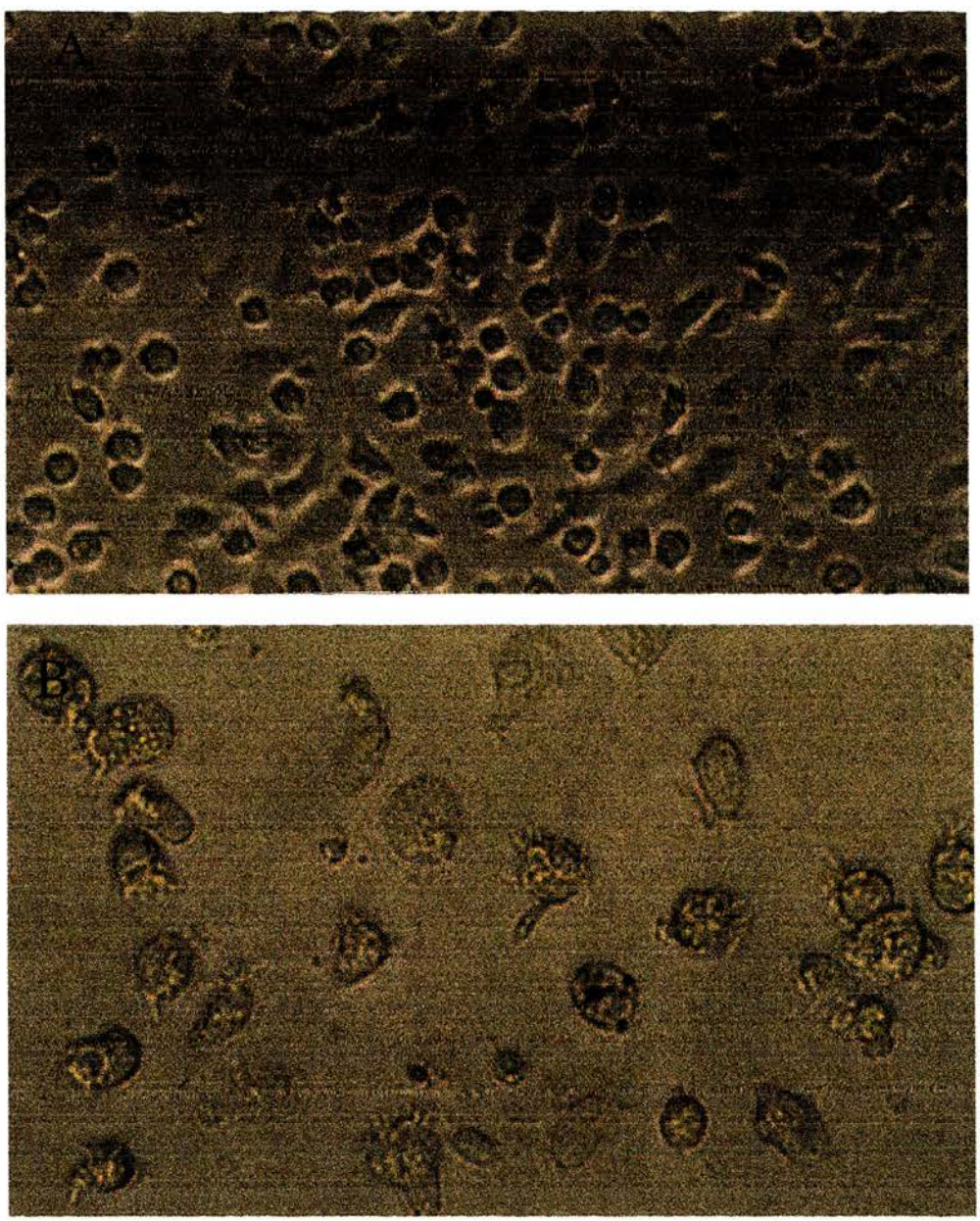


Figure 5.2 Effect of culture duration on susceptibility of monocytes to MVV infection as determined by PCR

Cells ($2x10^5$ cells /well) were exposed to MVV at 0.02 $TCID_{50}$ per cell on either day of isolation (day 0) or day7 in culture and harvested at 12h, 48h and 7 days after infection. The cell lysates were analysed by PCR for 35 cycles with primers to *pol* region, which amplify a 217 bp region of *pol* gene. PCR products were separated in 2% agarose and detected by Southern hybridisation. The figure is representative of three replicate experiments. P: positive control. MW: 1 kb DNA ladder.

A: MVV POL. Stained with ethidium bromide

B: Corresponding autoradiogram. Probed with ^{32}P labelled probe

Figure 5.3 Effect of culture duration on content of viral DNA synthesis in infected monocyte and MDMs

Cells were exposed to MVV at 0.02 $TCID_{50}$ per cell on the day 0 (day of isolation) or 1, 2 and 7 in culture and harvested at 24 hours after infection. Lysates were analysed with primers *LTR* (which amplifies a 156/116 bp region of LTR, *pol* (which amplifies a 217 bp region of *pol*) and *gag* (which amplifies a 461 bp region of *gag*). Cell equivalents ($2x10^5$) were used in each reaction. PCR products were separated by 2% agarose and stained with ethidium bromide. P. positive control, N: negative control containing no DNA, MW: 1kb DNA ladder.

Fig.5.2

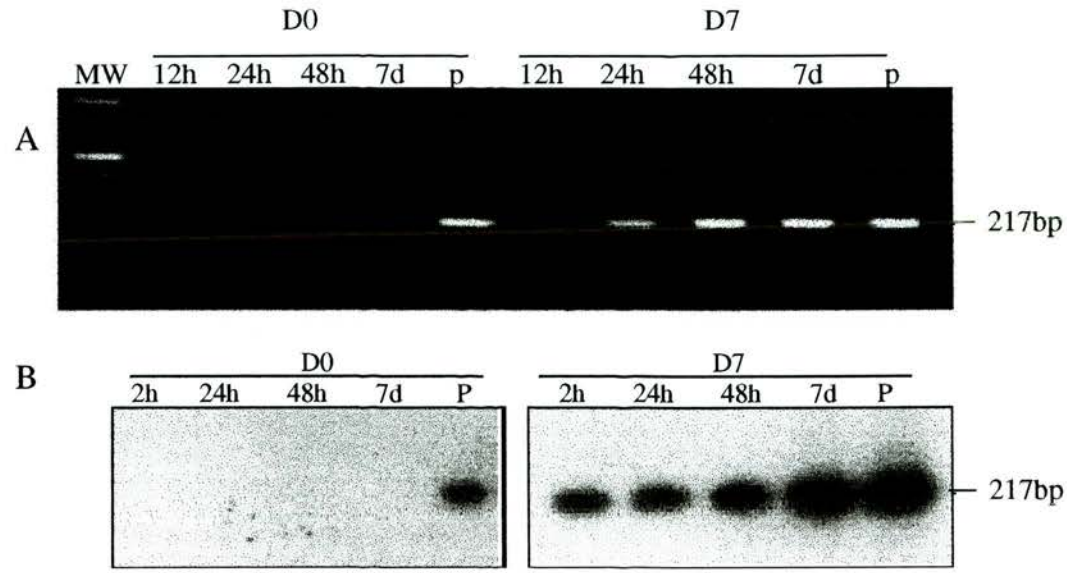
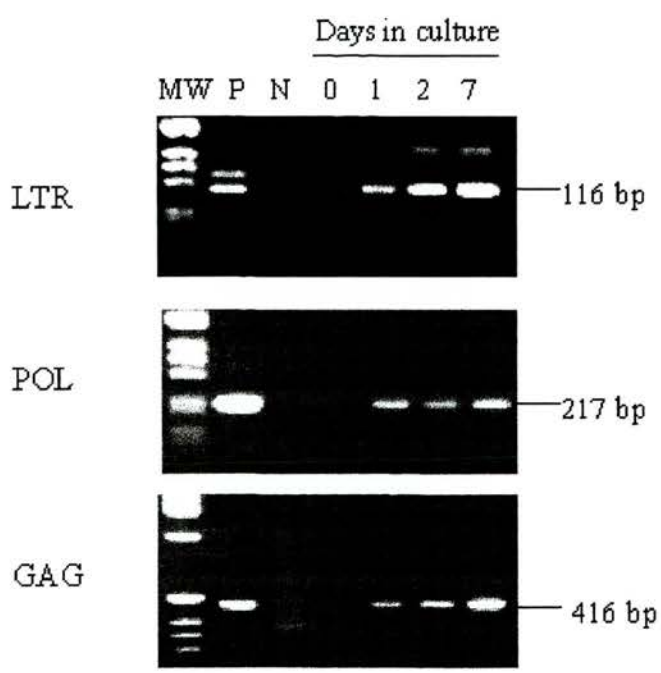

D0
D7
MW 12h 24h 48h 7d p 12h 24h 48h 7d p
A
217bp
B
D0
D7
2h 24h 48h 7d P 2h 24h 48h 7d P
217bp

Fig.5.3

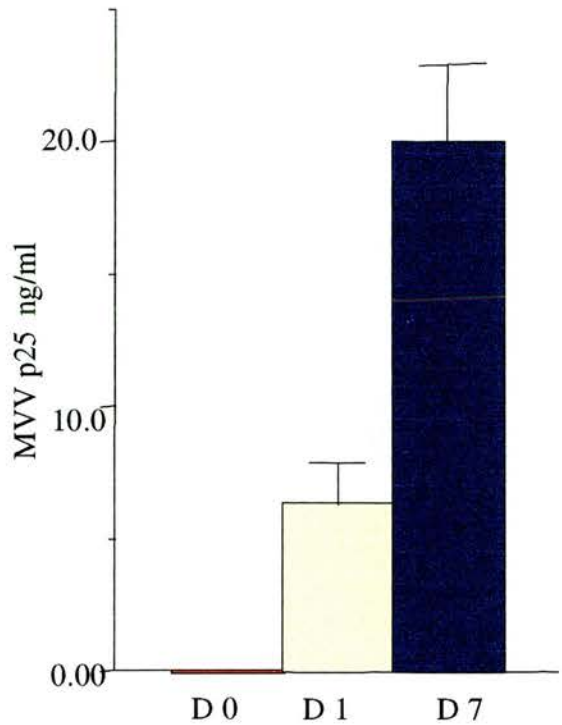

Days in culture
MW P N 0 1 2 7
LTR
116 bp
POL
217 bp
GAG
416 bp

concept that the monocyte is not susceptible to productive infection with MVV (Narayan *et al.* 1982).

Since viral DNA synthesis could not be detected in fresh monocytes, but was detectable within 12 hours of infection of MDMs, the next experiment was designed to investigate whether reverse transcription was initiated in monocytes exposed to MVV on the day of isolation using PCR with three primer sets. Cell lysates were analysed with *LTR*, *pol* and *gag* primers pairs to detect viral DNA initiated within the first 24 hours of infection. In three experiments, no products were detected in day 0 monocytes with any of the three sets of primers, indicating that reverse transcription had not been initiated in these cells. This result was not due to a lack of, or difference in amount of amplified DNA within these samples as ATPase primers gave similar levels of amplification with all samples (data not shown). On other hand, when cells were maintained for a day or more prior to infection, viral DNA were clearly detected within 24 hours of infection (Fig 5.3). Increasing time in culture before infection resulted in increasing amount of transcripts of LTR, *pol* and *gag* detectable at 24 hours.

5.3.2.2: **Levels of p25 antigen in MVV-infected fresh and cultured monocytes**

Virus replication was next analysed by measuring p25 antigens in culture supernatants by competitive ELISA at 14 days after infection. Data from three separate experiments were very similar and summarised in Fig.5.4. The results show that the levels of p25 antigen detected in monocyte culture increased with time in culture (Fig.5.4). No p25 antigen was detected in day 0 culture. However, p25 antigen was detectable in monocytes cultured for 1 or 7 days, but 7 day-old cells show higher virus replication, measured by p25 antigen than cells infected at day 1 (maturing macrophages). The level of p25 antigen increased with time in culture. This may be a reflection of the increase in cellular gene transcription and protein synthesis, which characterises the maturation process. The results above suggest that monocytes are susceptible to MVV infection at all stages of maturation, but the



Figuree 5.4 Levels of p25 antigen in MVV-infected 0-day-old, 1-day-old and 7-day-old monocytes, respectively

Cells were exposed to MVV at a 0.02 $TCID_{50}$ per cell on the day 0 (day of isolation) or dayl or day 7 in culture. Cell culture supernatants were harvested at 14 days post-infection and analysed by measuring p25 antigen in by a competitive ELISA as described in Materials and Methods (Chapter 2). Data were obtained by comparison of mean OD values (from triplicate wells) with a standard curve from recombinant MVVp25.

kinetics of virus replication depends on the stages of differentiation at the time of infection.

5.3.2.3: **Kinetics of MVV replication in macrophages**

The efficiency of replication of MVV EV1 strain was studied in MDMs from MVV seronegative sheep that were cultured 5 days after isolation, before investigating the effect of cytokines GM-CSF, TGF-β and IFN-γ on MVV replication. As determined by the p25 levels in culture supernatant, the replication of MVV in this culture system is easily demonstrated over 14- day time course (Fig.5.5).

5.3.3: **Effect of cytokines GM-CSF, TGF-β and IFN-γ on MVV replication in MDMs**

In initial experiments, the effect of exogenous cytokines GM-CSF, TGF-β and IFN-γ on MVV replication in MDMs was examined. MDMs were pre-treated with GM-CSF (10ng/ml), TGF-β (10ng/ml) and IFN-γ (20 ng/ml), respectively. The supernatants were harvested at day 7 post infection for measurement of viral p25. The cells were washed with PBS and lysed in DNA lysis buffer for quantification of viral DNA.

5.3.3.1: **Enhanced effect of GM-CSF on viral expression**

5.3.3.1a: **p25 production in GM-CSF treated MDMs**

The effect of GM-CSF on MVV p25 production was determined using a competitive ELISA. The level of viral p25 in culture supernatants of macrophages treated with GM-CSF (10ng/ml) 1 day before and continuously after MVV infection was significantly ($P< 0.01$) higher than those of an equal number of macrophages cultured in medium alone (Fig.5.6).

In further experiments, the effect of various doses of GM-CSF was examined with the cytokine level ranging from 100pg/ml and 100ng/ml. MVV replication was assessed at viral protein levels using the competitive ELISA. These studies show that the stimulatory effects of GM-CSF on MVV replication are concentration dependent

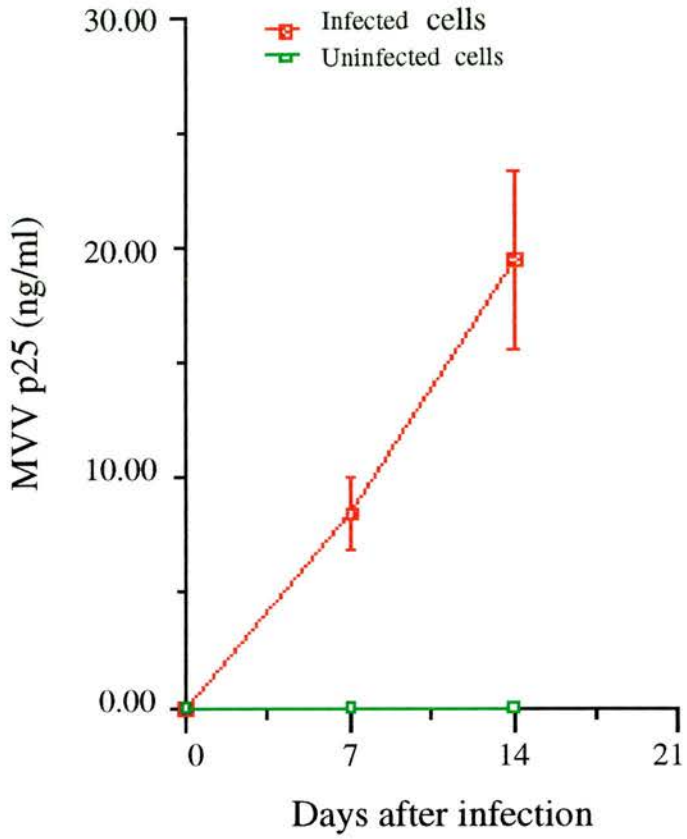


Figure 5.5 Kinetics of MVV replication in MDMs, as reflected by p25 level in culture supernatant

MDMs were infected with MVV EV1 strain at 0.02$TCID_{50}$ /cell, and supernatants were harvested at the indicated time points for p25 determination. Data were obtained by comparison of mean OD values (from triplicate wells) with a standard curve from recombinant MVVp25. Data are expressed in ng/ml as the mean ± SD from three individual experiments.

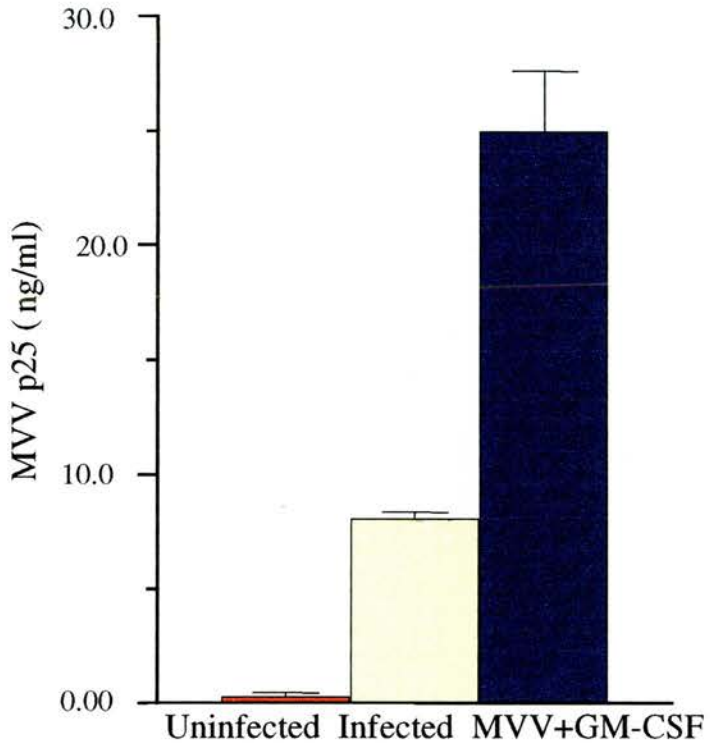


Figure 5.6 Effect of GM-CSF to stimulate MVV replication in MDMs

MDMs were prepared as described in Materials and Methods (Chapter 2) and cultured for 5 days, and seeded in 24-well plates (1 $x10^5$). Cells were incubated with GM-CSF (10 ng/ml) 1 day before and continuously after infection with MVV EV1 (0.02 TCID50 per cell). Cell-free supernatants were harvested on days 7 for p25 determination by using competitive ELISA. Data were obtained by comparison of mean OD values (from triplicate wells) with a standard curve from recombinant MVVp25.

in MDM (Fig.5.7). Increasing concentration of GM-CSF yielded an increasing stimulatory effect on MVV p25 expression over the range tested. Maximum stimulatory concentration was obtained at the concentration of 10 ng/ml (Fig.5.7).

To confirm that the observed stimulation was specifically due to GM-CSF, the GM-CSF preparation used was heat inactivated for 45 min at 56°C. Use of heat inactivated GM-CSF resulted in similar amounts of virus p25 production to untreated MDMs, but significantly less than those of GM-CSF treated MDMs (Fig.5.8), which confirms the specificity of stimulatory effect of GM-CSF on MVV replication and excludes non-specific effects.

5.3.3.1b: **Effect of GM-CSF on viral DNA expression**

Effect of GM-CSF on the replication of MVV in MDM was further examined by quantification of viral DNA using QC-PCR with a primer pair to the *pol* region. As shown in Fig.5.9, MVV replication (when measured 7days after infection) in MDMs treated with GM-CSF 1 day before and continuously after virus infection was markedly enhanced when compared to untreated MDMs. All amplification products were analysed by Southern blot hybridisation (Fig.5.9B) with a radiolabeled DNA probe specific for a *pol* sequence internal to the primer pair. Autoradiographs were analysed by an optical scanning system that assigns optical density values to each specific band on the autoradiograms. The amount of MVV DNA present in lysate of MVV infected MDMs was estimated from a standard curve derived from DNA of plasmid pPOL2 using QC-PCR and then expressed as amount per 500ng DNA. The quantitative evaluation of the PCR signal clearly shows that the level of viral DNA in GM-CSF treated MDMs was higher than that in untreated MDMs and increased with concentration of GM-CSF added (Fig.5.10). Comparable results were obtained in each of three different experiments.

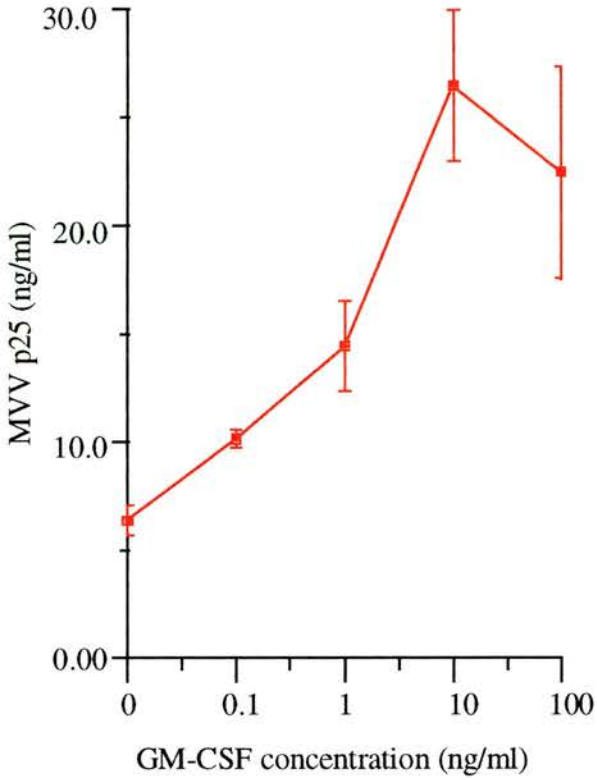


Figure 5.7 Dose-dependent stimulation of MVV replication in MDMs cultured in the presence of GM-CSF

MDMs were prepared as described in Materials and Methods, and seeded in 24-well plates (1 x10^5). Cells were incubated with GM-CSF (0.1, 1, 10, 100 ng/ml) 1 day before and continuously after infection with MVV EV1 (0.02 $TCID_{50}$ per cell). Culture supernatants were collected at day 7 after infection and assayed for level of viral p25 by ELISA. Data were obtained by comparison of mean OD values (from triplicate wells) with a standard curve from recombinant MVVp25. Each point is presented means ± SD of three replicates and representative of three independent experiments.

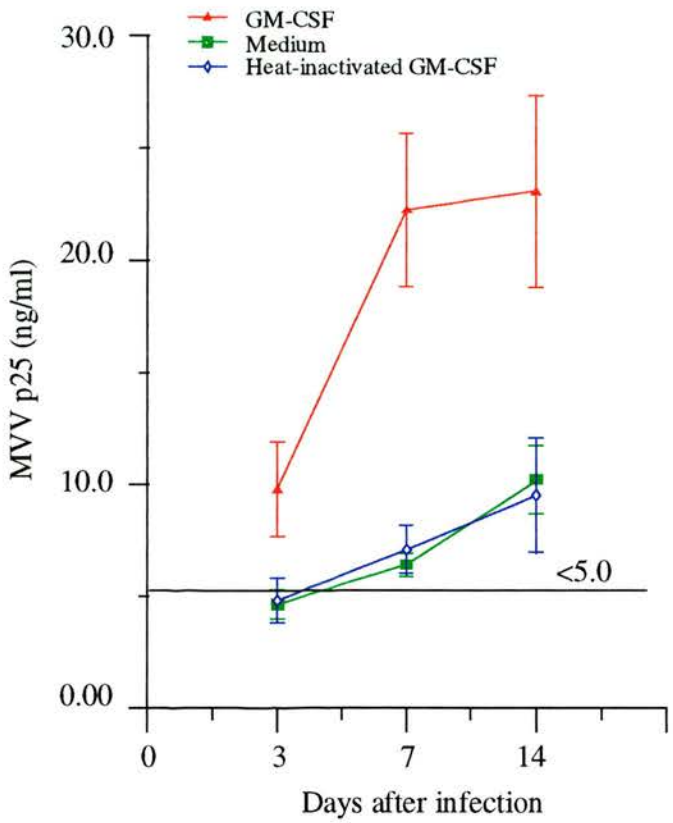


Figure 5.8 Heat inactivation of GM-CSF abrogated GM-CSF-mediated stimulation of MVV replication in MDMs

MDMs were prepared as described in Materials and Methods, and seeded in 24-well plates (1 x10^5). Cells were incubated with GM-CSF (10 ng/ml) or heat-inactivated GM-CSF (10ng/ml) or medium only 1 day before and continuously after infection with MVV EV1 (0.02 $TCID_{50}$ per cell) and culture supernatants were collected at the indicated time after infection. Results are presented means ± SD of three replicates and representative of three independent experiments. Data were obtained by comparison of mean OD values (from triplicate wells) with a standard curve from recombinant MVVp25.

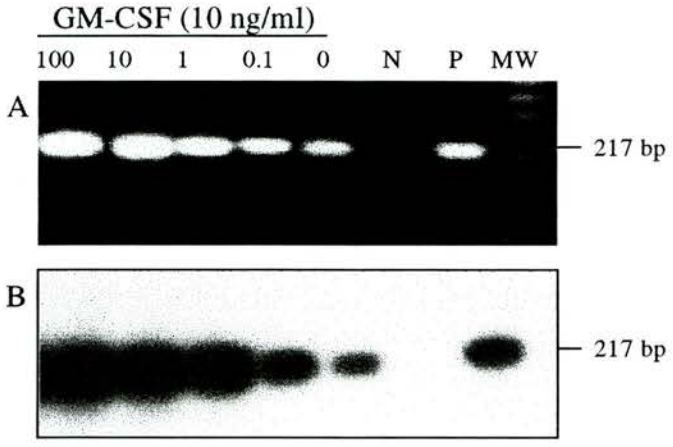
Figure 5.9 Levels of MVV DNA in lysates of MVV infected MDM treated with GM-CSF

MDMs were treated with a range (0 to 100ng/ml) of GM-CSF concentration added 1 day prior to virus challenge at 0.02 $TCID_{50}$/cell and subsequently maintained with GM-CSF. At 7 day after infection, 500ng of total DNA purified from cell lysates was analysed for levels of MVV DNA by PCR using primers specific for MVV *pol*. N: negative control. P: positive control. MW: 1kb DNA ladder

A: Stained with ethidium bromide

B: Probed with ^{32}P labelled internal probe.

Fig.5.9



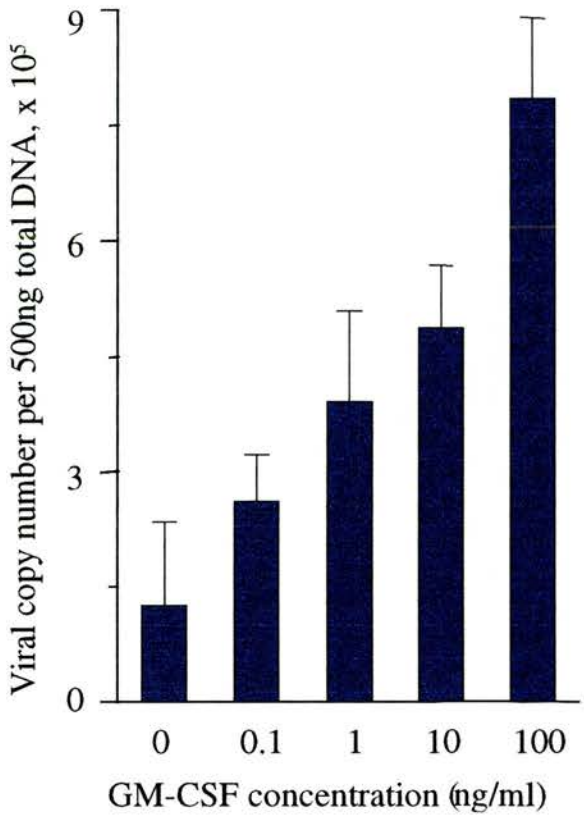


Figure 5.10 Levels of MVV DNA in lysates of MVV infected MDM treated with GM-CSF

MDMs were treated with a range (0 to 100ng/ml) of GM-CSF concentration added 1 day prior to virus challenge at 0.02 $TCID_{50}$/cell and subsequently maintained with GM-CSF. At 7 day after infection, DNA purified from cell lysates was analysed for levels of MVV DNA using QC-PCR with primers from *pol* gene. Autoradiographs were analysed by scanning densitometry. Data are presented in copy number/500ng total DNA as the mean (± SD) of triplicate assays for each treatment used.

5.3.3.1c: **Kinetics of GM-CSF induced stimulation of viral replication**

The kinetics of MVV production induced in MDM treated with GM-CSF is shown in Fig.5.11. The time-course studies were conducted to follow the observed GM-CSF induced stimulation of viral replication up to 14 days post-infection. This shows that enhanced MVV expression induced by GM-CSF was observed as early as 3-days post infection, and was still seen at 14 days post infection.

5.3.3.1d: **GM-CSF mediated induction of MVV RNA**

As shown in Fig.5.12, GM-CSF up-regulated MVV mRNA expression when GM-CSF was added 1 day before infection. An increased hybridisation signal for *pol* was observed in GM-CSF treated MDMs in comparison to an equal number of untreated MDM using RT-PCR. Therefore, GM-CSF has effect in stimulating MVV RNA production

5.3.3.2: **Inhibitory effect of TGF-β of on MVV replication in infected MDMs *in vitro***

5.3.3.2a: **Effect of TGF-β on extracellular MVV production by macrophages**

As shown in Fig.5.13 TGF-β significantly decreased the amount of extracellular MVV p25 produced in MDMs culture compared with that found in untreated-MVV infected cultures ($P<0.05$, Student's t test).

5.3.3.2b: **Dose-dependent response of TGF-β on MVV expression**

In further experiments, the effect of various doses of this cytokine was examined with cytokine level ranging from 100pg/ml to 100ng/ml. Viral levels were measured by RT-PCR (Fig.14A) and QC-PCR (Fig.14B). The modulatory effects of TGF-β on MVV replication are concentration dependent in MDMs and increasing concentrations of TGF-β yielded increasing inhibition of MMV replication over the range tested (Fig.14). TGF-β was consistently active in inducing inhibition of MVV expression when tested from four different sheep.

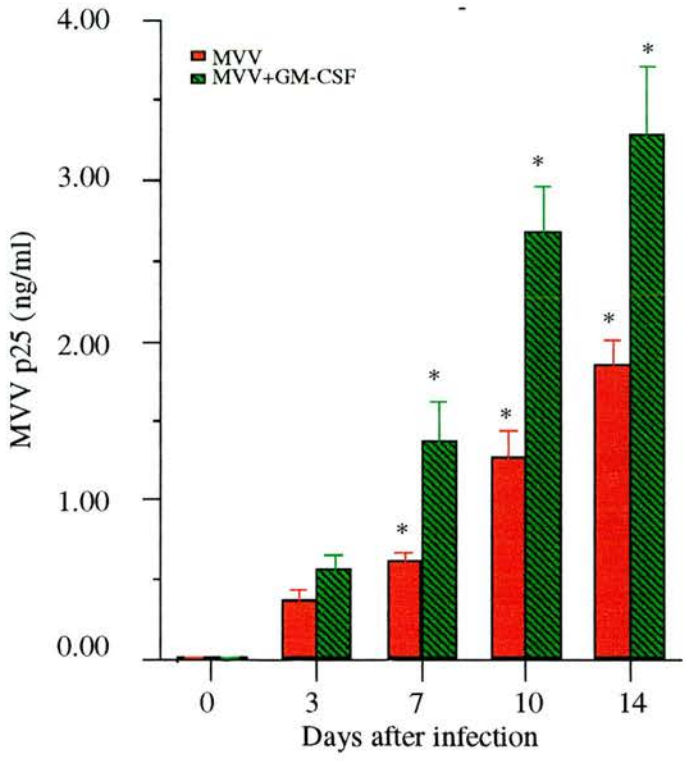


Figure 5.11 Kinetics of GM-CSF induced stimulation of viral replication

ELISA values for extracellular p25 antigen levels over 14 days post-infection (MVV EV1 0.02 $TCID_{50}$/cell. Cultures treated with GM-CSF (10 ng/ml) for 1 day before and continuously after MVV infection show a continued stimulation of viral output. Data are expressed in ng per millilitre as the mean + SD from triplicate assays for each treatment. Statistically significant differences ($P < 0.05$, Mann-Whitney non-parametric statistics) between results with treatment of GM-CSF and those without treatment of GM-CSF are denoted by asterisks.

Figure 5.12 GM-CSF mediated induction of MVV RNA

Cells were exposed to MVV at 0.02 $TCID_{50}$ per cell and harvested at 24 hours after infection. Lysates were analysed with primers POL4 and POL5 (which amplifies 217 bp region of *pol*) by RT-PCR. Cell equivalents ($2x10^5$) were used in each reaction. PCR products were separated on 2% agarose and detected by Southern hybridisation. To control for input RNA in samples analysed for MVV cDNA. PCR with ATPase primers was performed at $2x10^5$ cell equivalent. The 167bp amplification was separated on 2% agarose and stained with ethidium bromide.

A: MVV POL

B: ATPase

Lanes 1 and 2: treatment with GM-CSF.

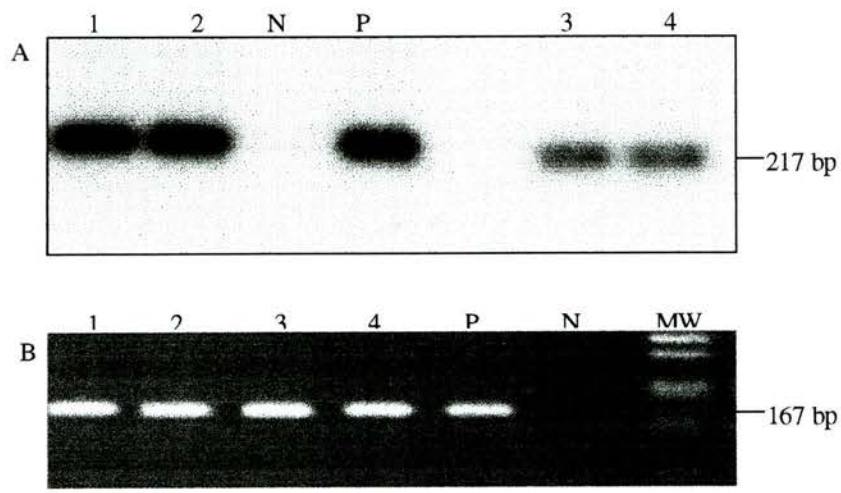
Lane 3 and 4: medium only.

P: positive control.

N: negative control containing no RNA

MW: 1kb DNA ladder

Fig.5.12



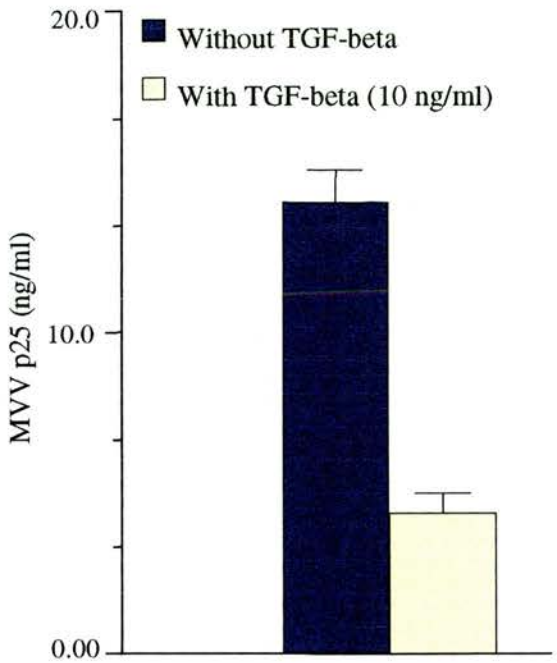


Figure 5.13 Inhibitory effect of TGF-β on MVV replication in MDMs

MDMs were prepared as described in Materials and Methods, and seeded in 24-well plates (1 x10^6). Cells were incubated with TGF-β (10 ng/ml) 1 day before infection with MVV EV1 (0.02 $TCID_{50}$ per cell) and incubated with complete medium containing TGF-β. Cell-free supernatants were harvested on days 6 for p25 determination by using ELISA. Data were obtained by comparison of mean OD values (from triplicate wells) with a standard curve from recombinant MVVp25. Results shown are the mean (± SD) of three separate experiments. Standard deviations (SD) are represented by error bars. There are statistically significant differences ($P < 0.05$, Mann-Whitney non-parametric statistics) between results with MVV infected cells with treatment of TGF-β and those without treatment of TGF-β.

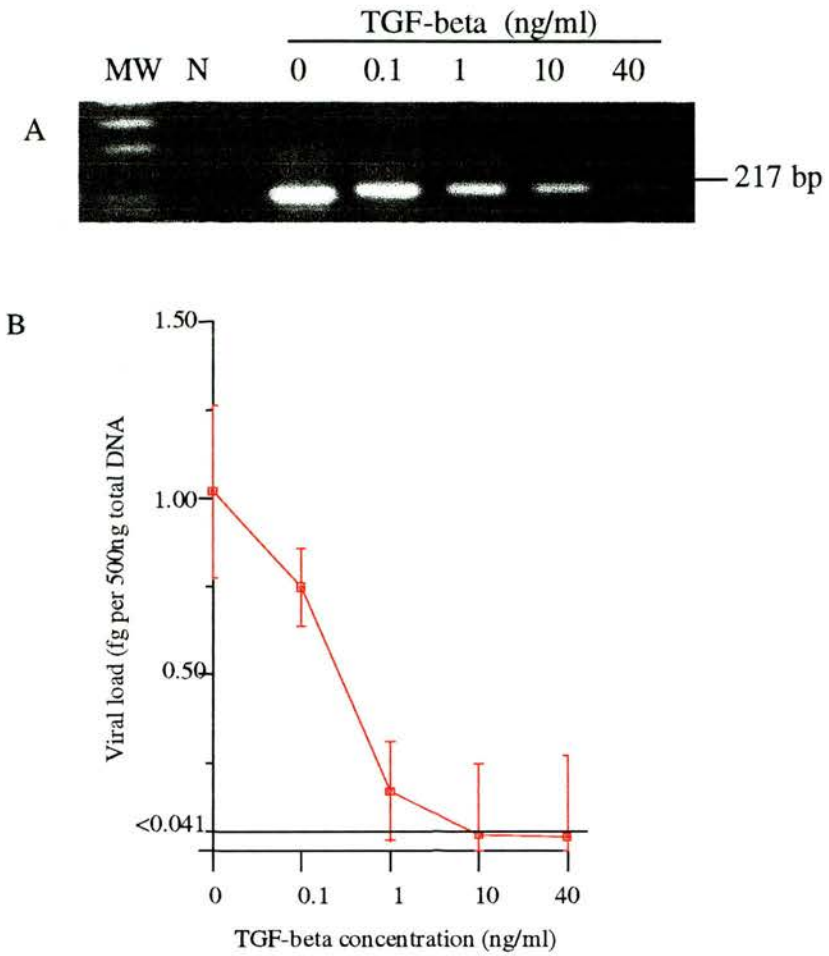


Figure 5.14 Dose-dependent inhibition of MVV replication in MDMs cultured in the presence of TGF-β

MDMs were prepared as described in Materials and Methods, and seeded in 24-well plate (1 x10^6). Cells were incubated with TGF-β (0.1, 1, 10, 40 ng/ml) 1 day before and continuously after infection with MVV EV1 (0.02 $TCID_{50}$ per cell). Infected cells were collected at day 3 after infection. Viral levels were measured by RT-PCR for viral RNA (A) and by QC-PCR for viral DNA (B). Each point is presented as means ± SD of three replicates and representative of three independent experiments.

5.3.3.2c: **Kinetics of TGF-β- induced inhibition of viral replication**

The kinetics of MVV production induced in MDMs pre-treated with the cytokine is shown in Fig.5.15. The time-course studies were conducted to follow the observed TGF-β-induced inhibition of viral replication up to 14 post-infection by ELISA. Detection of p25 antigen in culture supernatant by ELISA showed that inhibition of MVV expression induced by TGF-β was observed at 7 days after infection and was still seen at 14 days after infection.

To confirm that the observed inhibitory effect was specifically due to TGF-β, the TGF-β preparation used was heat-inactivated for 60 min at 65°C. Use of heat inactivated TGF-β resulted in similar amounts of virus p25 production (Fig.5.15), viral DNA (Fig.5.16) and RNA level (Fig.5.17) to untreated MDMs, but significantly higher ($P<0.05$) than those of TGF-β treated MDMs (Fig.5.15 and 5.16). For comparison, TPA was used as stimulator of virus replication. TPA at a concentration of 1ng/ml has been shown to be very effective in stimulating viral RNA expression in blood monocytes (see Chapter 3). These findings confirm the specificity of the inhibitory effect on MVV replication and exclude non-specific effects.

5.3.3.2d**: TGF-β delays and inhibits MVV transcription kinetics**

In investigating the potential mechanisms of anti-MVV action of TGF-β, the kinetics of transcription were compared between TGF-β-treated and untreated MDMs by using PCR amplification of LTR The results show that a 4 to 8 hours delay of appearance of viral RNA in TGF-β-treated cells in comparison with untreated MDM (Fig5.18). The MVV LTR RNA was detectable by 4 hours and 8 hours in untreated and treated MDMs respectively.

5.3.3.2e: **TGF-β acts as a modulator in MDM**

In investigating the effects of TGF-β (10ng/ml) on infected MDM, the recovery of virus after removal of cytokine was examined. Cessation of treatment with cytokine at day 7 after infection was followed by a further 7 days of culture, and then the

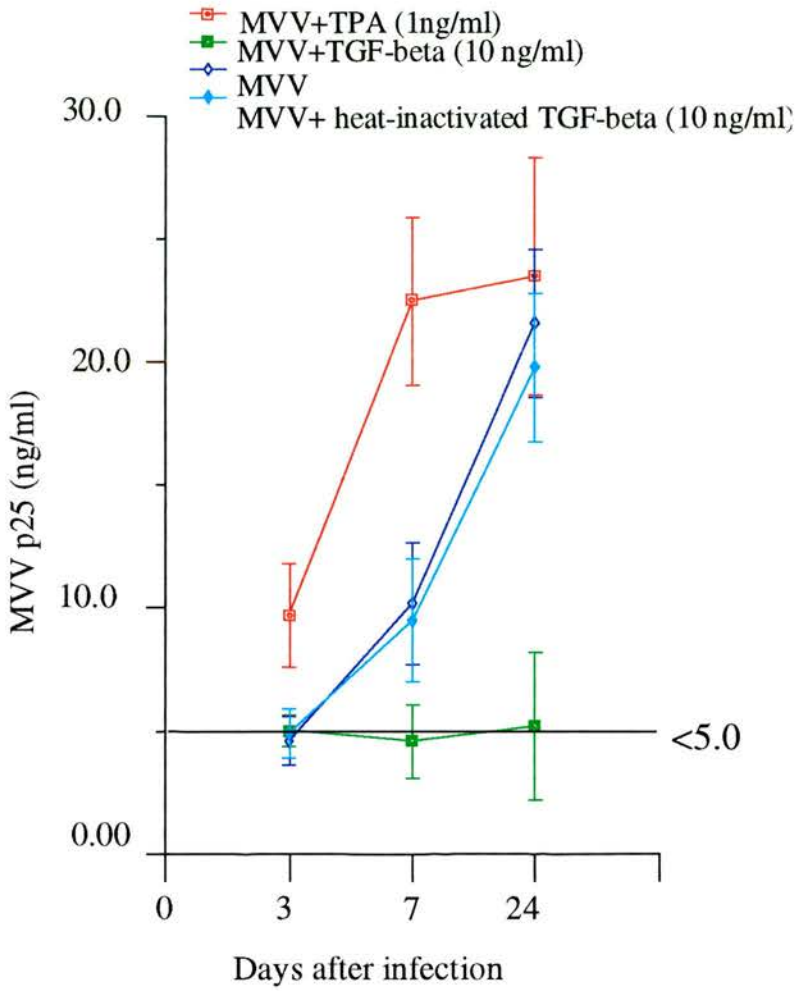


Figure 5.15 Kinetics of TGF-β induced inhibition of viral replication

MDMs were prepared as described in Materials and Methods, and seeded in 24-well plate (2 x10^5). Cells were incubated with TGF-β (10ng/ml), heat-inactivated TGF-β(10ng/ml), TPA (1 ng/ml) medium only 1 day before and continuously after infection with MVV EV1 (0.02 $TCID_{50}$ per cell). Culture supernatants were collected at 3, 7 and 14 days after infection and assayed for the level of viral p25 as described in Chapter 2. Cultures treated with TGF-β (10 ng/ml) shows a continued inhibition of viral output. Data are expressed in ng per millilitre as the means ± SD from triplicate assays for each treatment. Data were obtained by comparison of mean OD values (from triplicate wells) with a standard curve from recombinant MVVp25.

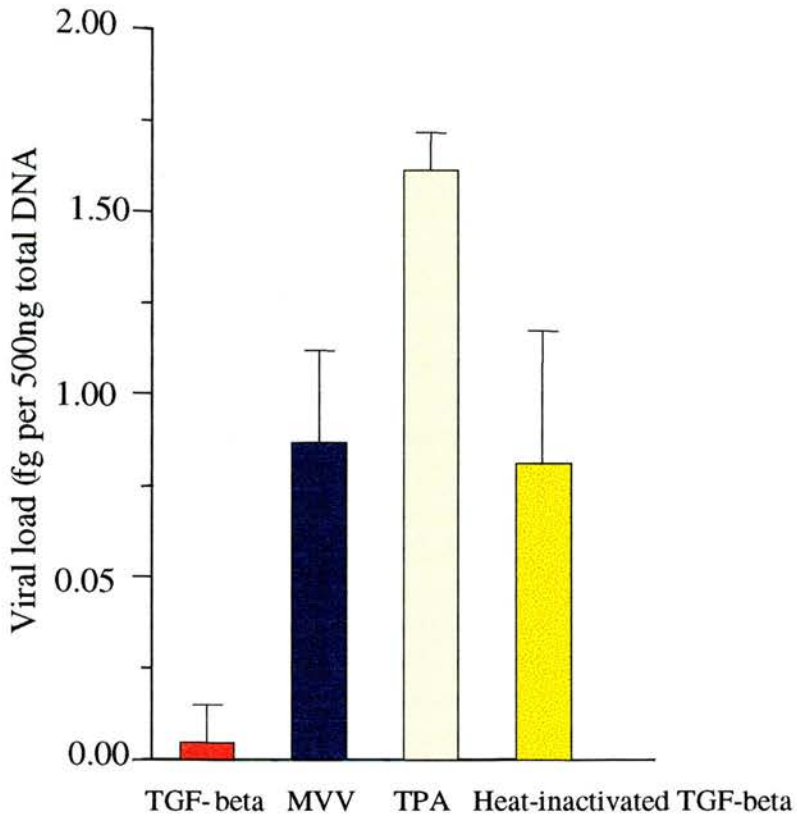


Figure 5.16 Heat inactivation of TGF-β abrogated TGF-β-mediated inhibition of MVV DNA expression in MDMs

MDMs (2 x10^5) were incubated with TGF-β (10 ng/ml), heat-inactivated TGF-β (10ng/ml), TPA (1 ng/ml) and medium only 1 day before and continuously after infection with MVV EV1 (0.02 $TCID_{50}$ per cell). Culture supernatants were collected at 3 days after infection and assayed for the level of viral DNA by QC-PCR. Results are presented as means ± SD of three replicates and representative of three independent experiments. Standard deviations (SD) are represented by error bars. There are statistically significant differences ($P < 0.05$, Student's t test) between results with TGF-β treated MVV-infected cells and those without treatment of TGF-β or with treatment of inactivated TGF-β.

Figure 5.17 Heat inactivation of TGF-β abrogated TGF-β-mediated inhibition of MVV replication in MDMs

Cells (2 x10^5 cells/well) were incubated with TGF-β (10 ng/ml), heat-inactivated TGF-β (10ng/ml), TPA (1 ng/ml) and medium only 1 day before and continuously after infection with MVV EV1 (0.02 $TCID_{50}$ /cell). Infected cells were collected at 3 days after infection and assayed for the level of viral RNA by RT-PCR.

Lane1: treatment with TGF-β (10 ng/ml).
Lane 2:heat-inactivated TGF-β (10ng/ml).
Lane 3: medium only
Lane 4: TPA (1 ng/ml).

Figure 5.18 TGF-β delays and inhibits MVV transcription

MDMs were prepared as described in Materials and Methods, and seeded in 24-well plates (2 x10^5 cells/well). MDMs were incubated with TGF-β (10 ng/ml) and medium only 1 day before and continuously after infection with MVV EV1 (0.02 $TCID_{50}$ per cell). Cells were collected at the indicated time after infection and assayed for the level of viral RNA by RT-PCR using the primers specific for LTR sequence. Results are representative of three independent experiments.

Fig.5.17

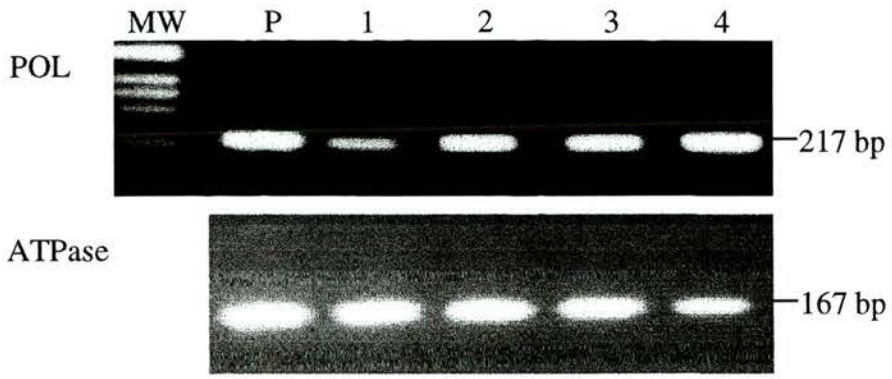
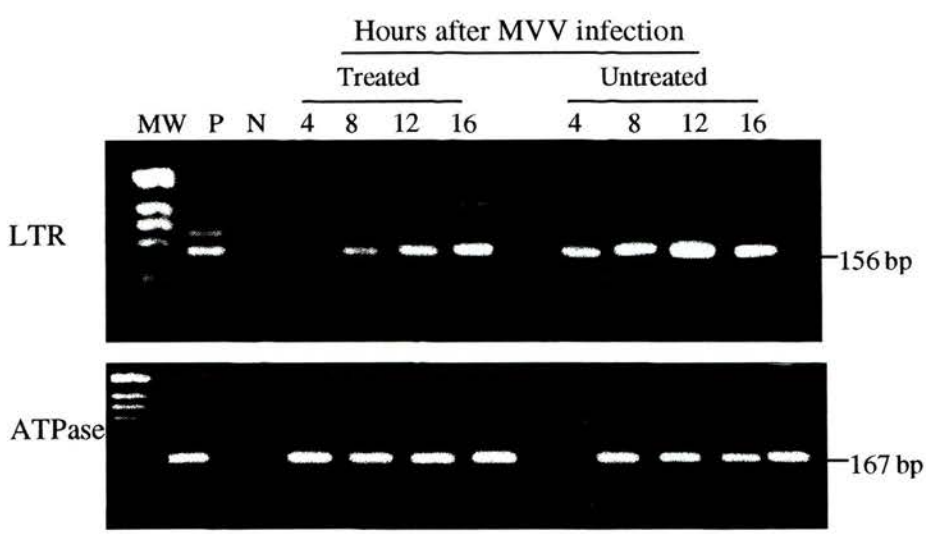


Fig.5.18



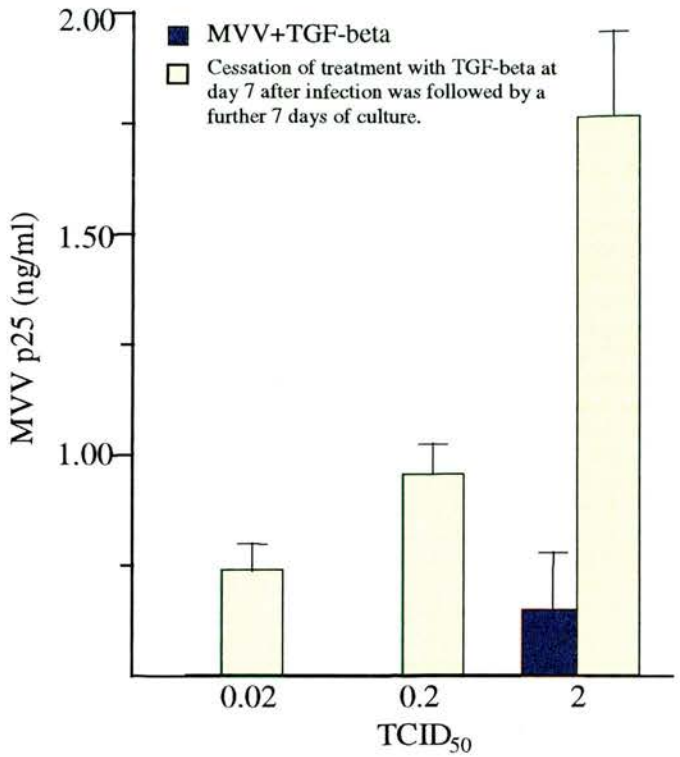


Figure 5.19 TGF-β acts as a modulator in MDMs

MDMs were prepared as described in Materials and Methods, and seeded in 24-well plates (2 x10^5). Cells were incubated with TGF-β(10 ng/ml) and medium only 1 day before and continuously after infection with MVV EV1 (0.02, 0.2 and 2 $TCID_{50}$ per cell, respectively). Cessation of treatment with TGF-β at the day 7 after infection was followed by a further7 days of culture and then culture supernatants were harvested, in which recovery of virus production was measured by an ELISA. Results are expressed in ng/ml as the mean ± SD from triplicate assays for each treatment.

culture supernatant was harvested. Viral production was then measured by p25 ELISA. The recovery of virus production was proportional to the initial $TCID_{50}$ of MVV used. Infected MDM treated with TGF-β for 7 days and cultured further 7 days without treatment of TGF-β yielded a higher level of virus than those which had been continuously treated with TGF-β (Fig.5.19, $P < 0.05$, Mann-Whitney non-parametric statistics).but lower than those untreated control (data not shown). These findings suggest that TGF-β may act as a modulator of a latent state after infection.

5.3.3 3: **MVV replication *in vitro* is not affected by IFN-γ**

The level of MVV replication in MDM treated with IFN-γ 1 day before and continuously after infection was determined at protein and nucleic acid level by using p25 ELISA and PCR, respectively. There was no significant variation in the level of p25 expression, compared with that in untreated MDM (Fig.5.20A). Similarly, the level of viral DNA measured by QC-PCR (Fig.5.20B) and viral RNA measured by RT-PCR (Fig.5.20C) was unaffected in MDM by IFN-γ treatment.

Figure 5.20 MVV replication *in vitro* is not affected by IFN-γ

MDMs were prepared as described in Materials and Methods, and seeded in 24-well plates (2 x10^5 cells/well).

A: IFN-γ does not affect production of MVV p25 antigen in MDMs.

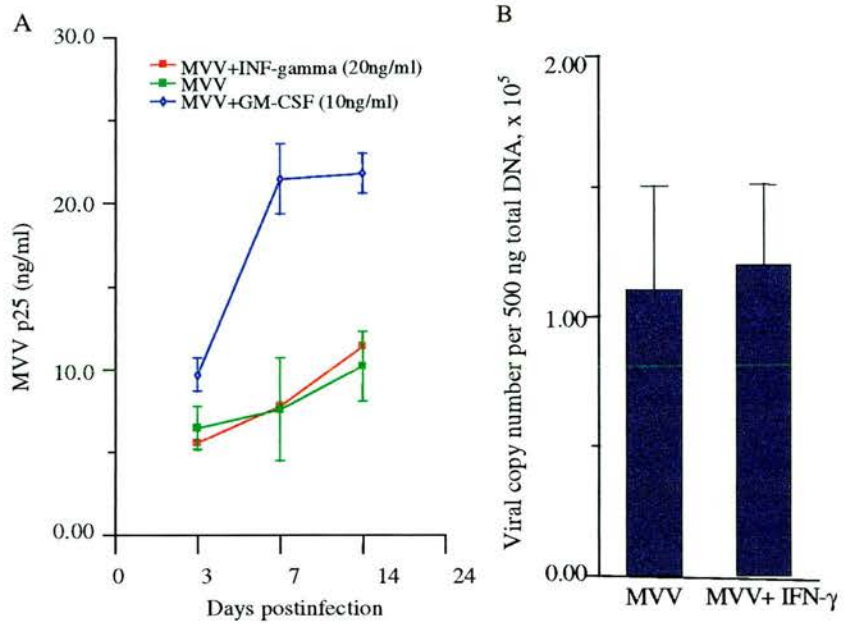
Cells were incubated with IFN-γ (20 ng/ml), GM-CSF (10ng/ml) and medium only 1 day before and continuously after infection with MVV EV1 (0.02 $TCID_{50}$ per cell). Infected cells were collected at 7 days after infection and assayed for the level of viral antigen p25 by ELISA as described in Chapter2. Results are presented as means ± SD of three replicates and representative of three independent experiments.

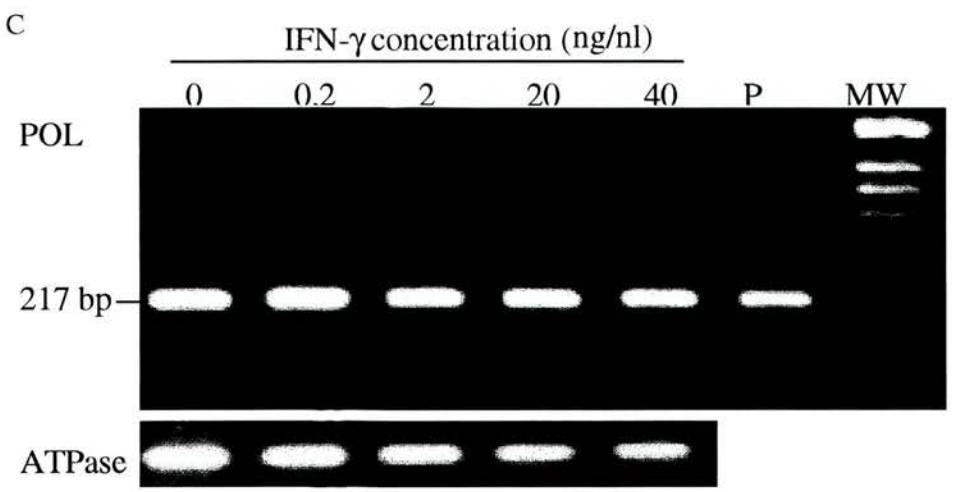
B: Effect of IFN-γ on expression of MVV DNA in MDMs.

Cells were incubated with IFN-γ (20 ng/ml) and medium only 1 day before and continuously after infection with MVV EV1 (0.02 $TCID_{50}$ per cell). Culture supernatants were collected at 7 days after infection and assayed for the level of viral DNA by QC-PCR as described in Chapter 3. Results are presented as means ± SD of three replicates.

C: Effect of IFN-γ on expression of MVV RNA in MDMs.

Cells (2 x10^5 cells/well) were incubated with IFN-γ (0, 0.2, 2, 20, 40 ng/ml) 1 day before and continuously after infection with MVV EV1 (0.02 $TCID_{50}$ per cell) and were collected at 7 days after infection and assayed for the level of viral RNA by RT-PCR as described in Chapter 2. Results are representative of three independent experiments.


A
30.0
20.0
10.0
0.00
MVV p25 (ng/ml)
MVV+INF-gamma (20ng/ml)
MVV
MVV+GM-CSF (10ng/ml)
0
3
7
14
24
Days postinfection
B
2.00
1.00
0.00
Viral copy number per 500 ng total DNA, x 10^5
MVV
MVV+ IFN-γ


C
IFN-γ concentration (ng/nl)
0
0.2
2
20
40
P
MW
POL
217 bp
ATPase

CHAPTER 5:

Discussion

5.4.1: **MVV replication is blocked in freshly isolated peripheral blood monocytes.**

The role of monocyte/macrophages in the pathogenesis of MVV infection is incompletely understood. MVV virion, viral DNA or RNA and viral protein have been identified in monocytes/macrophages found in peripheral blood, lung and a wide variety of tissues (Narayan *et al.* 1982, 1985, 1985, Gendelman *et al.* 1985, 1986, Gorrell *et al.*, 1992, Brodie *et al.* 1995). In lung, the detection of viral proteins or infectious virus in infected target cells is associated with lymphoid infiltration (Brodie *et al* 1995). In view of these data and the tropism of many lentiviruses for cells of the monocytes/macrophages lineage, it has been proposed that these cell have the potential to act as a reservoir of MVV and to disseminate virus to other cells within the infected host. In monocytes, viral replication is highly restricted to a minimal rate (Narayan *et al.* 1982), often only small quantities of viral RNA production occurs and undetectable concentration of capsid protein is synthesised (Brahic *et al.* 1981). High rate of viral replication only occurs once these cells mature to macrophages (Narayan *et al.* 1985). Infection of monocytes appears to be linked, at least in part, to differentiation (Narayan *et al.* 1985), i.e. the restriction of maturation of the infected cells limits the replication of virus. In this respect, MVV resembles many lentiviruses viral which RNA transcription and protein synthesis is closely linked to the rate of maturation of monocytes to macrophages (Kalter *et al.* 1991).

In this study, it has been demonstrated that monocytes exposed to MVV immediately upon isolation from peripheral blood do not become productively infected. It was found that, in fresh monocytes, there was no evidence of viral DNA synthesis initiated up to 7 days after infection (Fig.5.2).

It was reported previously that viral RNA was detectable soon after isolation of fresh

monocytes form infected animals but virus replication did not extend beyond low levels of transcription of viral RNA (Gendelman *et al* 1986). Although reverse transcription did not occur after the infection of fresh monocytes with MVV *in vitro*, it was found that a short period in culture was sufficient for reverse transcription to proceed (Fig5.3) and for the cells to be productively infected. In fact, as little as 1 day in culture was sufficient for transcripts to be detected. If the cells were left to mature for longer periods before infection with MVV, the infection becomes more efficient, with an increase in viral load in cells according to the intensity of specific bands on the blot. In MDMs infected with MVV, the transcripts were detected soon after exposure to MVV. These results agree with the widespread observation that the macrophage is the principle cell type permissive for MVV replication in tissues (Gendelman *et al.* 1985, 1986, Gorrel *et al.*, 1992, Brodie *et al.* 1995) and also concur with reports that in human monocytes which have been maintained in culture for a day or more after isolation and purification from peripheral blood, HIV is able to bind, enter and reverse-transcribe its RNA *in vitro* (Kalter *et al.* 1991).

The result in this study that MVV replication is blocked in freshly isolated peripheral blood monocytes raises the question of whether monocytes in peripheral blood are susceptible to MVV infection *in vivo*. The *in vitro* finding in the study suggests that they are not and that examination of this compartment alone may not lead to an insight into the pathogenesis of MVV infection. This finding further supports the work of Gorrell *et al.* (1992) who found that MVV has a greater tropism for dendritic cells than monocytes.

5.4.2: **Effects of cytokines on MVV replication in MDMs**

MVV replicates and causes pulmonary disease amidst a cellular milieu that is likely to be rich in cytokines (Ellis *et al.* 1988, 1991). Therefore, precise characterisation of cytokine effects on MVV replication would be valuable in order to delineate the role of cytokines in lentiviral pathogenesis. In this study, the effect of the recombinant cytokines GM-CSF, TGF-β and IFN-γ on the replication of MVV in an *in vitro* model system was investigated.

5.4.2.1: **Stimulatory effects of GM-CSF on MVV replication in MDMs**

As GM-CSF is a key regulator of monocyte-macrophages and is required for the survival, proliferation, and differentiation of monocyte-macrophages and for the function of their mature progeny (Methcalf *et al.* 1992, Crowe and Lopez 1997). Furthermore, the ability of monocytes-macrophages to harbour the MVV and to permit its replication suggests that cytokines which affect production of monocytes, their differentiation and functional status, may modulate MVV replication. Based on this concept, the effect of GM-CSF on MVV replication in MDMs *in vitro* was explored. The results demonstrate that GM-CSF can up-regulate MVV replication in primary infected MDMs. To determine the effects of GM-CSF on MVV replication in macrophages, cells were treated with GM-CSF from 24 hours before infection with different concentrations of cytokine. The effect of the GM-CSF on viral replication varies with the cytokine dose. Concentrations higher than 0.1 ng/ml enhanced MVV replication in macrophages. The specificity of this stimulatory effect was confirmed by use of heat-inactivated GM-CSF.

The above results demonstrating the enhancing effect of GM-CSF on MVV replication in MDMs are consistent with those of Perno *et al.* (1989) with HIV in monocytes and Koyanagi *et al.* (1988) with HIV in primary mononuclear phagocytes. Furthermore, these results parallel those of Folks *et al.* (1987), who demonstrated the induction by GM-CSF of HIV-1 replication in chronically infected immature monocytic cell lines. Collectively these studies are at variance with others that reported inhibitory effects of GM-CSF on lentivirus expression. Matsuda *et al.* (1995) reported an inhibitory effect of GM-CSF on the replication of HIV in human blood MDMs. Moreover, the studies of Hammer *et al.* (1986) indicated that GM-CSF could suppress viral replication in a promonocytic cell line chronically infected with HIV. The reasons for the discrepancies are not clear, but may relate to the target cell population used or dose and duration of cytokine treatment or virus strains studied. Nevertheless, the results in this study support the concept that GM-CSF could be an important mediator driving the virus replication, thus promoting virus spread.

The results in this study demonstrated of GM-CSF up-regulates the expression of MVV RNA and DNA in MDMs, indicating that this cytokine may exert its effects at various stages during MVV replication. Increased amount of viral DNA was observed in MDM pretreated with GM-CSF, suggesting that GM-CSF pretreatment of MDM stimulates early steps of MVV replication between absorption and reverse transcription. The effect on viral DNA synthesis may be important enough for accounting for a part of the increased RNA level. IFN-α has been reported to have no effects on HIV transcripts that were clearly independently from the effect on viral DNA in the early steps of the life cycle (Meylan *et al* 1993). It is also possible that the level of viral RNA increased in MDM pretreated with GM-CSF, suggesting an additional effect on transcription. MVV transcription directed by the 5'LTR is regulated by the interplay of viral proteins such as Tat and host cell factors. Therefore GM-CSF may augment MVV replication in MDM is through stimulation of the production host transcription factors, which bond to the MVV LTR, resulting in transactivation and thus in an increase in MVV transcription. The increase in the MVV RNA in GM-CSF treated macrophages reflects the effect of GM-CSF on multiple rounds of MVV infection of macrophages. Further experiments are required to measure single-step growth curves using the high concentration of MVV as inoculom to define further the role of GM-CSF in MVV infection. In addition, differentiation of monocyte/macrophages by GM-CSF may result in the increase permissiveness to MVV infection. This differentiation-induced susceptibility to infection is true for MVV, where replication has been found to depend on the state of maturation and differentiation of the infected cells (Gendelman *et al* 1986). Viral gene expression may vary up to more than ten-fold between PBM and AM in MVV infected sheep (Chapter3). Similarly, even in absence of exogenous cytokines, the result in the study has demonstrated the relative lack of permissiveness of freshly isolated monocytes to MVV infection and dramatically increased susceptibility to MMV infection of MDM (Fig.5.2), further suggesting that cellular permissiveness of cells of macrophages lineage to infection may alter during the maturation process. Another possible way that GM-CSF argument the expression of viral RNA and DNA is that GM-CSF can induce proliferation of monocyte/macrophages

(Schuitemaker *et al* 1990). It is conceivable that GM-CSF might increase the amount of MVV through increased proliferation of the treated, infected cells. This has been studied carefully in cases of HIV infection. Koyanagi *et al.* (1988) demonstrated that exposure to GM-CSF resulted in up to 100-fold enhancement in HIV replication as assessed by quantification of HIV p24 antigen. Walsh *et al.* (1992) have also demonstrated in SIV that in GM-CSF-treated cells there were more cells containing gag protein than in cells not exposed to GM-CSF, suggesting that cytokine treatment may cause an increase in the percentage of infected macrophages.

Of note is the fact that it has been found that the level of GM-CSF mRNA was enhanced in MVV-infected macrophages *in vivo* (Chapter 4) and hyperevelation of GM-CSF in MVV-infected lung (Woodall *et al.* 1997) suggesting that *in vivo* AM might a main potential producer of GM-CSF, which stimulates the virus replication.

5.4.2.2: **Inhibitory effects of TGF-β on MVV replication in MDMs**

TGF-β comprises a group of molecules that are produced during the normal response to injury (Rappolee *et al.* 1988). The TGF-β produced locally triggers a series of events ranging from chemotactic attraction of monocytes to induction of gene expression for several cytokines including the autocrine regulation of TGF-β (Wahl *et al.* 1991). To explore the effects of TGF-β on MVV replication in macrophages, cells were treated with TGF-β from 24 hours before infection with decreasing concentrations of cytokine. The inhibitory effect on viral replication was achieved at concentrations that are comparable to those required to induce other effects on macrophages (Wahl *et al.* 1991). This inhibitory effect is specific for TGF-β, since it was confirmed by use of heat-inactivated TGF-β (Fig5.16). It was also found to be related to a reduction in the rate of transcription and dependent on the continued presence of TGF-β as demonstrated by virus recovery experiments after removal of TGF-β (Fig.5.19). This suggests that pre-treatment of TGF-β decreased the number of successfully infected MDMs *in vitro*. These results are consistent with the observation that in HIV-infected macrophages, TGF-β suppresses HIV replication

(Poli *et al.* 1991). This is in contrast to the work of Lazdins *et al.* (1991) who found a stimulatory effect on MVV replication. It is possible that differences in culture methods and condition, time of treatment with cytokine may explain the discrepancy between our findings and those of other groups, although which differences contribute most of these discrepancies in MVV infection is still unclear.

Many steps have been defined in MVV infection and replication, such as binding to the cell surface, internalisation of the viral core into the cytoplasm, uncoating of viral RNA, reverse transcription, integration of viral DNA to cellular DNA, viral DNA transcription and translation, viral components assembly and virus budding. In this study it was found that that TGF-β delays and inhibits MVV transcription suggesting a possible mechanism by which TGF-β might inhibit viral replication at transcriptional level. Further study is required to clarify this point. Another possible explanation worth investigating could be that TGF-β exerts a negative influence on the expression of critical cellular proteins involved in the replication of MVV, such as enzymes of the cell signalling pathway that ultimately impact on nuclear translocation or other cellular factors which might downregulate other surface molecules needed for virus binding or inhibit cellular components involved in virus fusion, uncoating or transcription. This effect is different from that of IL-13 which inhibits HIV production independently of effects on the total amount of DNA (Montanier *et al.* 1993). However, it does not exclude an effect of TGF-β on an alternative antiviral mechanism. Taken together these findings implicate TGF-β as a candidate mediator of MVV latency within macrophages *in vivo*.

The above described effects of TGF-β, taken together with the observations described in Chapter3 and 4, suggest that TGF-β provides a possible mechanism by which macrophages can suppress MVV replication *in vivo* and inhibit the spread of infection by creating an inhibitory system. Finally, since others cytokines capable of inducing expression of MVV, such as GM-CSF (Chapter 5) have been found be increased in AM (Chapter 4) or lung tissue (Woodall *et al.* 1997), the balance

between stimulatory and inhibitory cytokines may play a critical role in controlling MVV replication and expression and subsequently in the clinical progression of MVV infection.

5.4.2.3: **MVV replication *in vitro* is not affected by IFN-γ**

Previous work has shown the presence of an interferon species (lentiferon) with biochemical characteristics of both IFN-α and IFN-γ in culture of leukocytes from the lung of lambs with experimental MVV infection (Lairmore *et al.* 1988a) or of macrophages from goat blood cells exposed to CAEV (Narayan *et al.* 1985). This IFN present in cultures derived from lambs with lymphocytic interstitial pneumonia had moderate inhibitory effect on MVV replication *in vitro* (Lairmore *et al.* 1988a). The levels of IFN produced by pulmonary leukocytes correlated with an increase in virus replication in tissue (Lairmore *et al* 1988a).

In the present study, it was not possible to demonstrate any apparent enhanced or inhibitory effects of IFN-γ on MVV replication in MDMs. The results demonstrated that IFN-γ does not influence MVV replication in MDMs (Fig.5.20). The same phenomenon has been observed in other virus infection. For example in hepatitis delta virus (HDV), the replication of HDV in transfected cell lines has been shown to be not affected by IFN-γ despite intact cellular responses to interferons and dsRNA (McNair *et al.* 1994).

The reasons for the discrepancies are not obvious, but may relate to the target cell population used or dose and duration of cytokine treatment or virus strains studied and/or the particular origin of IFN may determine what effects the cytokine has on lentiviral replication. It should be considered that *in vitro* culture of blood monocytes results in a time-dependent differentiation into macrophages characterised by an increased expression of certain surface markers (such as CD11/CD18 and CD14 antigens) (Gessani *et al.* 1993, Lee *et al.* 1997). This differentiation process can result in marked change in cell behaviour such as an increased susceptibility to MVV itself (Narayan *et al.* 1982) and loss of ability to expression of IL-10 mRNA in

response to MVV infection (Chapter 4), which may significantly affect virus replication. It is reasonable to assume that differences in the these experiment including the differentiation stage of macrophages on the time of virus infection or addition of cytokines, can exert marked influence on the results and contribute to some of these conflicting data available on the role of endogenous cytokines in the replication of MVV infection in monocytes/macrophages.

5.4.3: **Conclusion**

The results of the present study showed that GM-CSF and TGF-β have contrasting effects on MVV replication. GM-CSF stimulates and TGF-β suppresses the MVV replication in macrophages. IFN-γ exerts no effect on MVV replication. From these data it is speculated that these cytokines may alter MVV expression in macrophages. However, because of the complex network of cytokine interactions it is difficult to draw clear-cut conclusions on the roles of specific cytokines in MVV replication in macrophages. Further experiments with neutralising antibody to cytokines should be carried out to understand the role of a given specific cytokine. Further studies are required to demonstrate conclusively the involvement of cytokines in modulating the replication of MVV *in vivo*.

CHAPTER 6

CYTOKINE MODULATION OF CELL SURFACE MOLECULES ON MACROPHAGES

6.1: **Introduction**

The expression of cell surface molecules on macrophages is an important marker of their functional activity. It has been shown that these molecules on monocytes/ macrophages were modulated during lentiviral infection (Rossen *et al.* 1989, Pantaleo *et al.* 1991, Fecondo *et al.* 1993, Luj□ *et al.*1993, 1994, Berman *et al.* 1994a). It has been shown that HIV infection resulted in alterations in the level of expression of surface molecules of the infected monocyte (Autran *et al.* 1988, Davidson *et al.* 1988, Wright *et al.* 1988, Twigg *et al.* 1989, Vermot-Desroches *et al.* 1990, Agostini *et al.* 1992, Stent *et al.* 1994). In MVV infection, Luj□ *et al.*(1993) showed that AM in MVV-infected sheep expressed an increased level of MHC class II. Because MHC class II acts as a component of cellular receptor for MVV (Dalziel *et al.* 1991), the increased expression of the cell surface of the receptor for MVV may favour the infection of AM, thus suggesting that cell surface molecules might be good candidates to explain the involvement of monocytes/ macrophages in pathogenesis of lentiviral infection. However, the mechanisms responsible for modulation of cell surface molecules on infected monocytes/ macrophages are poorly understood. It has been hypothesised that changes in the expression of cell surface molecules may be directly related to viral infection of the cells. It is also possible that the virus stimulates cells to release various cytokines and other factors which may in turn alter the expression of molecules. It has been shown that HIV infection, by stimulating the production of IL-1β and TNF-α, leads to autocrine feedback with production of other cytokines such as IL-6 and GM-CSF (Merrill *et al.* 1991, Longo *et al.* 1993). In MVV infection, elevated expression of GM-CSF mRNA was observed in AM of MVV infected sheep (Chapter 4). Cytokines or combinations of cytokines have been reported to regulate the expression of cell surface molecules such as MHC class I and class II (Nash *et al.* 1992, Schwartz 1992, Sallusto *et al.* 1994, Kruger *et al.* 1996, Thomssen *et al.* 1996,

Lee *et al.* 1997), CD14 (Kruger *et al.* 1996), CD1a, b, c molecules (Kasinrerk *et al.* 1993) on human monocytes and other leukocytes. Taken together, it is likely that changes on expression of cell adhesion molecules on monocytes are in part directly related to cytokines. However, there is little information on the effect of cytokines on modulation of the expression of cell surface molecules on sheep macrophages. There is therefore a clear need to understand the direct effect of cytokine on sheep macrophages.

CHAPTER 6:

Methods

6.2.1: **Assessment of surface antigens on cytokine-stimulated MDM**

6.2.1.1: **Antibodies**

Eight mouse monoclonal antibodies (McAbs) against sheep cell surface molecules were used for immunolabelling (Table 6.1). The secondary antibody alkaline phosphatase (AP) or FITC-conjugated goat anti-mouse IgG was purchased from Sigma.

6.2.1.2: **Detection of cell surface molecules on monocyte-derived macrophages**

Phenotypic characterisation of membrane antigens expressed on monocyte-derived macrophages (MDMs) was determined by a indirect immunofluorescence assay (flow cytometry analysis).

6.2.1.2a: **Flow cytometry**

MDM cells were pre-treated with cytokines 1 day before infection, leaving an equal portion uninfected or untreated. McAbs to cell surface CD11b, CD14, CD1b, MHC class I, MHC class II DR, MHC class II DQ, CD45c, CD11c (Table 6.1.2) were used to evaluate cell surface expression of these antigens by flow cytometry using a FACScan Cell Analyser (Becton Dickinson, San Jose CA). Cells were washed with PBS/ 1% BSA and stained with FITC-conjugated goat anti-mouse Ig (Sigma), as described in Chapter 2.

Table 6.1 Monoclonal anitbodies used in this study

Clone	Specificity	Dilution	Reference
VPM54	MHC class II (DR gene)	1:2	Dutia *et al.* 1993
VPM36	MHC class II (DQ gene)	1:2	Dutia *et al.* 1993
VPM19	MHC class I	1:2	Hopkins *et al.* 1990
VPM18	CD45	1:2	Hopkins *et al.* 1990
VPM65	CD14	1:2	Gupta et al 1996
IL-A15	CD11b	1:2	Gupta et al 1995
OMI	CD11c	1:2	Gupta et al 1995
CC-13	CD1b	1:2	Dutia *et al.* 1991

CHAPTER 6:

Results

6.3.1: **Effects of cytokines in MHC antigens expression**

In the following experiments the ability of ovine GM-CSF and bovine IFN-γ to modulate MHC antigens expression on ovine macrophages was examined *in vitro*. MDMs were recovered 3 days after treatment with the cytokine, stained with McAbs against MHC class I, MHC class II DQ and DR. Reactivity was assessed by flow cytometry

GM-CSF-stimulated MDMs were found to be positive for MHC class I antigens, but negative for MHC class II DR and DQ (Fig 6.1). In comparison with unstimulated cells, incubation of GM-CSF with MDMs increased the expression of MHC class I (Fig.6.2).. In contrast, GM-CSF showed no influence on the expression of MHC class II DR and DQ (Fig.6.1).

To investigate whether GM-CSF synergizes with MVV in induction of MHC class I, comparison of MHC class expression on GM-CSF-stimulated/uninfected and MVV-infected MDMs was carried out. As shown in Fig 6.3, the level of expression of MHC class I by GM-CSF stimulated/infected MDMs was decreased when compared with GM-CSF stimulated/uninfected MDMs. GM-CSF-stimulated/MVV-infected cells showed a lower fluorescence intensity when compared with GM-CSF stimulated/ uninfected MDMs, suggesting GM-CSF does not synergize with MVV in induction of MHC class I.

IFN-γ -stimulated MDMs were found to be positive for MHC class II DR and DQ as well as MHC class I (Fig 6.4). As shown in Fig.6.5, stimulation of IFN-γ did lead to a significant increase in the level of expression of MHC class II expression on MDMs when compared with control.

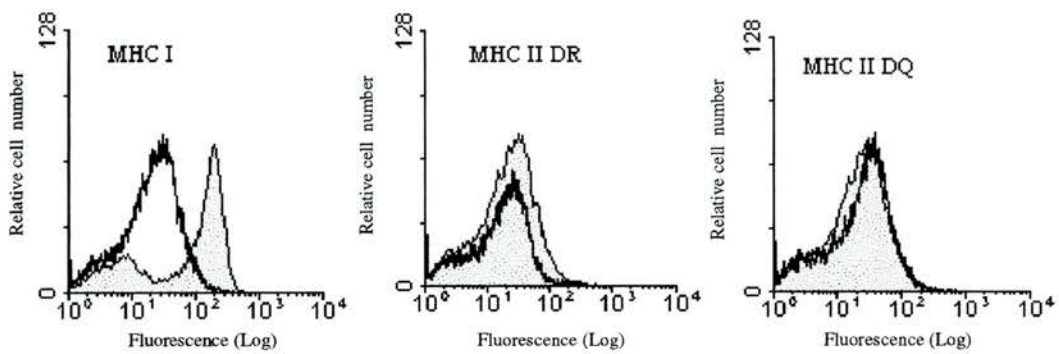


Figure 6.1 Flow cytometric analysis of MHC molecules on GM-CSF-stimulated macrophages

MDMs was cultured in the presence of GM-CSF (10ng/ml) for 3 days and then analysed by flow cytometry. Negative control antibody is shown as the blank. These data are representative of experiments that were repeated three times.

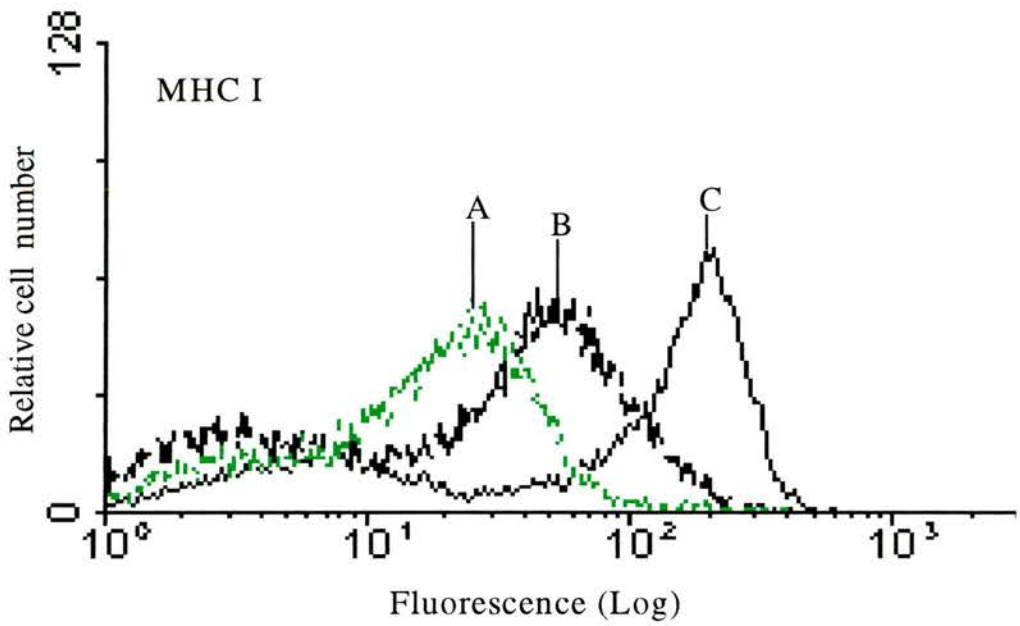


Figure 6.2 Flow cytometric analysis of MHC molecules on GM-CSF-stimulated and unstimulated macrophages (MDMs)

MDMs was cultured in either the presence of or absence of GM-CSF (10ng/ml) for 3 days and then analysed by flow cytometry. These data are representative of experiments that were repeated three times. A: negative controls, B: unstimulated cells, C: GM-CSF-stimulated cells.

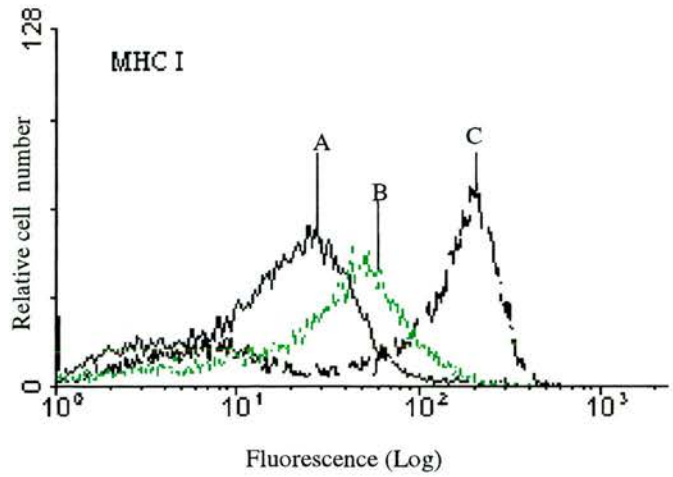


Figure 6.3 Flow cytometric analysis of MHC class I molecules on CM-CSF-stimulated/infected and uninfected macrophages (MDMs) with MVV *in vitro* (0.05 $TCID_{50}$ per cell)

MDMs were cultured in the presence of GM-CSF (10ng/ml) for 3 days after infection with MVV, then analysed by flow cytometry. These data are representative of experiments that were repeated three times. A: negative controls. B: GM-CSF + MVV. C: GM-CSF

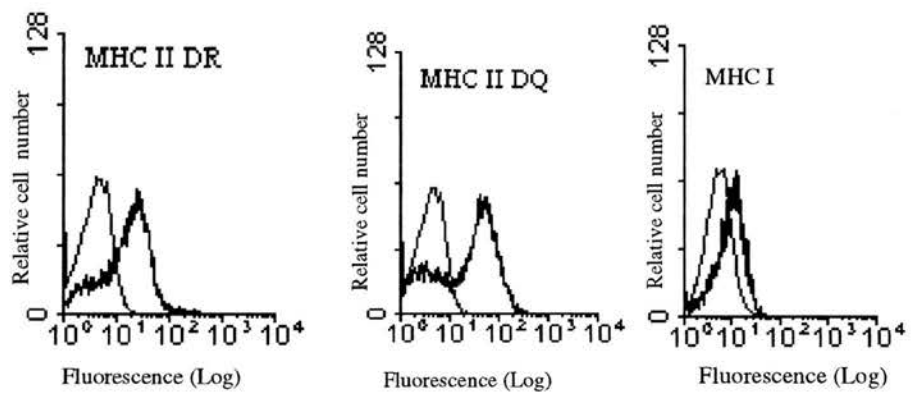


Figure 6.4 Flow cytometric analysis of MHC class I and II on macrophages stimulated with IFN-γ *in vitro*

MDMs were cultured in the presence of IFN-γ (10ng/ml) for 3 days and then analysed by flow cytometry as described in materials and methods. Negative control antibody is shown as the fine line. These data are representative of experiments that were repeated three times.

As shown in Fig 6.5, the level of expression of MHC class II DR by IFN-γ stimulated/infected MDMs was decreased with lower fluorescence intensity when compared with IFN-γ stimulated/uninfected MDMs. The level of expression of MHC class II DQ by IFN-γ -stimulated/MVV-infected was similar to that on IFN-γ stimulated/ uninfected MDMs.

6.3.2 **The effects of cytokines on CD antigens on macrophages**

In the following experiments the ability of ovine GM-CSF and bovine IFN-γ to modulate CD antigens expression on macrophages was examined *in vitro*. Cells were recovered 3 days after treatment with the cytokine, stained with McAbs against CD1b, CD11b, CD11c, CD14 and CD45. Reactivity was assessed by flow cytometry.

MDMs, treated either GM-CSF (Fig.6.6 and 6.7) or IFN-γ (Fig.6.8), were found to be positive for the surface molecules CD11c, CD14, CD45, but negative for CD11b, CD1b. However, treatment of either GM-CSF or IFN-γ did not lead to a significant increase in the level of expression of CD14 and CD45 on MDMs when compared with control. No significant in difference in the percentage of cells CD14 and CD45 was observed between stimulated and unstimulated cells.

Either GM-CSF (Fig.6.7) or IFN-γ (Fig.6.8) in combination with MVV had not any further enhanced effect on the expression of CD14 and CD45 on MDMs. Both cytokines showed no effects on the expression of CD1b and CD11b and CD11c.

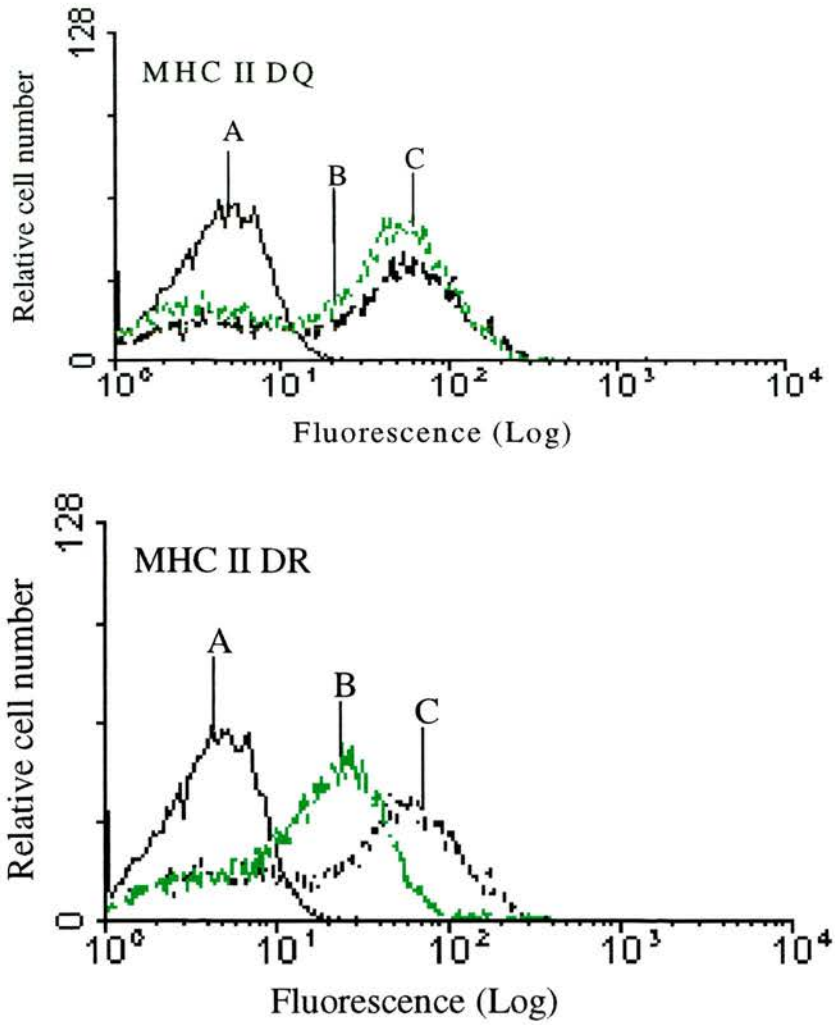


Figure 6.5 Flow cytometric analysis of MHC class II molecules on MVV-infected and uninfected macrophages (MDMs) stimulated with IFN-γ *in vitro*

MDMs were infected with MVV (0.02 $TCID_{50}$ per cell) and cultured in the presence of IFN-γ (10ng/ml) for 3 days, then analysed by flow cytometry. These data are representative of experiments that were repeated three times. A: negative controls. B: IFN-γ stimulated/infected cells. C: IFN-γ-stimulated/uninfected cells.

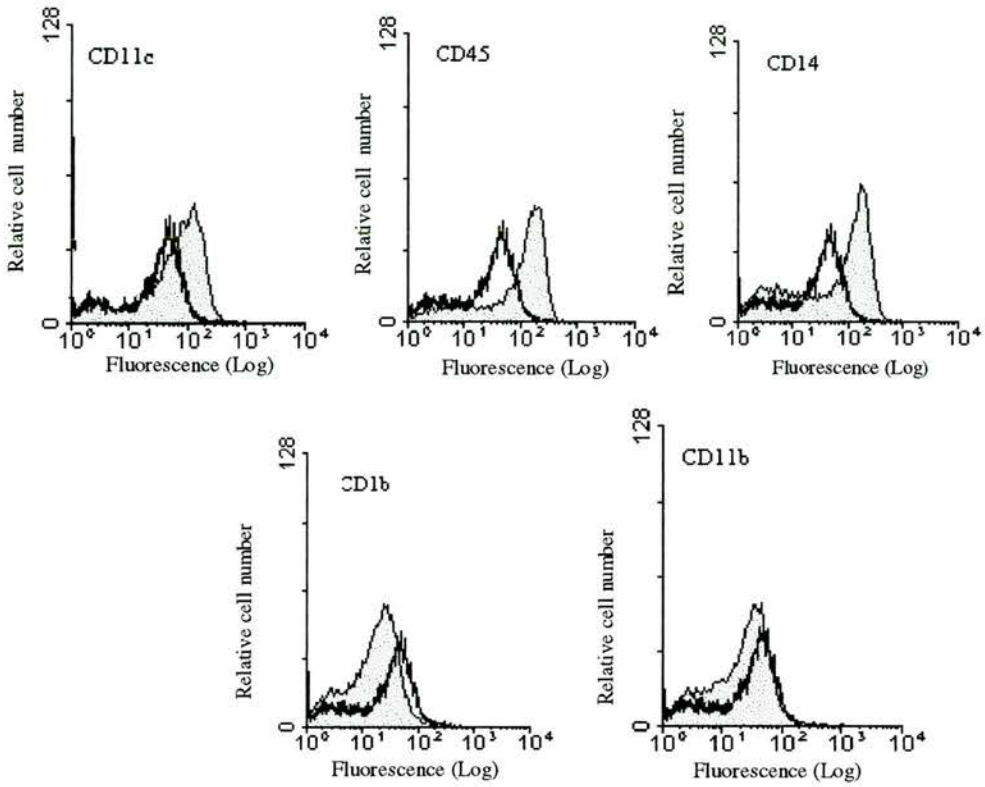


Figure 6.6 Flow cytometric analysis of the expression of CD14, CD1b, CD11b, CD11c and CD45on macrophages stimulated with or without GM-CSF

MDMs were stimulated with or without GM-CSF (10ng/ml) for 3 days and then analysed by flow cytometry as described in materials and methods. Negative control antibody is shown as the blank.

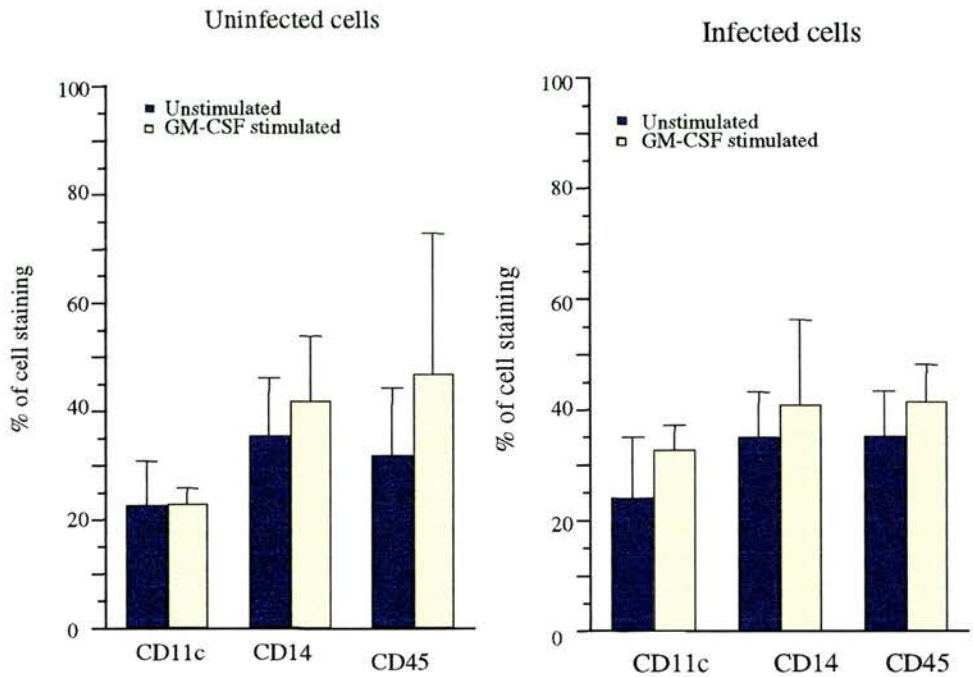


Figure 6.7 Comparison of CD11c, CD14 and CD45 on uninfected or infected macrophages stimulated with or without GM-CSF *in vitro*

MDMs was infected with or without MVV (0.02 $TCID_{50}$ per cell) and cultured in either the presence or absence of GM-CSF (10ng/ml) for 3 days, and then analysed by flow cytometry as described in materials and methods. Data are presented as mean ± SD of the percentages of macrophages expressing CD antigens.

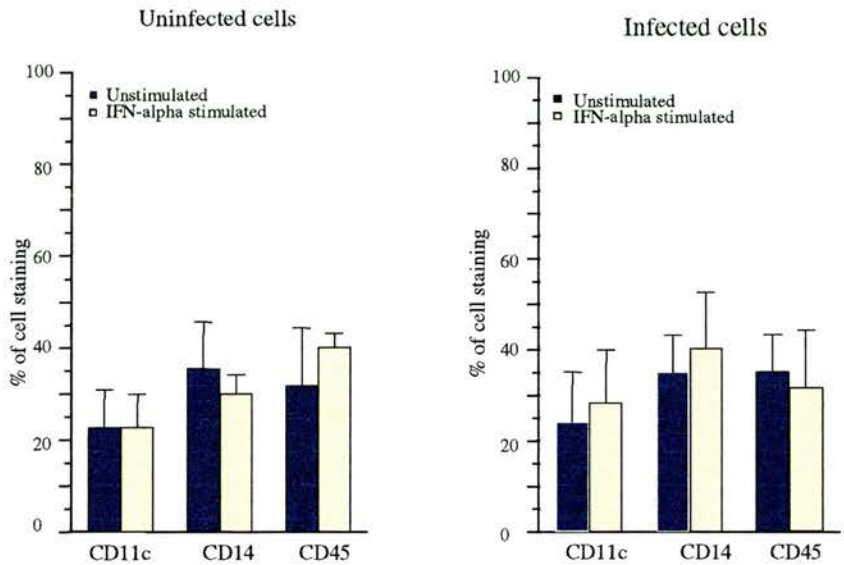


Figure 6.8 The expression of CD11c, CD14 and CD45 on uninfected or infected macrophages stimulated with or without IFN-γ *in vitro*

MDMs were infected with or without MVV (0.02 $TCID_{50}$ per cell) and cultured in either the presence or absence of IFN-γ (10ng/ml) for 3 days, and then analysed by flow cytometry as described in materials and methods. Data are presented as median ± SD of the percentages of macrophages expressing CD antigens.

CHAPTER 6:

Discussion

It is unclear what are the precise mechanisms involved in modulation of cell surface molecules in infected macrophages. It has been hypothesised that the virus might stimulate cells to release various cytokines and other factors that may in turn alter surface molecule expression. Several studies have shown in *in vitro* cell systems, cytokines or a combination of cytokines to modulate expression of antigen presenting (MHC class I and II) and co-stimulatory molecules on the surface of monocytes and other leukocytes (Mentzer *et al.*1986, Springer *et al.*1986, Te Velde *et al.*1988, Freyer *et al.*1988, Gimcher *et al.*1992, Jiang *et al.*1992, Nash *et al.*1992, Schwartz 1992, Sallusto *et al.*1994, Kruger *et al.*1996, Thomssen *et al.*1996, Lee *et al.*1997). Thus it is likely that changes in the expression of cell surface molecules on monocytes are in part related to cytokines. To test this hypothesis, MDMs were treated with either GM-CSF or IFN- γ.

Treatment with IFN-γ was able to augment the macrophage expression of MHC II. In human and murine models, IFN-γ has been demonstrated to be a potent mediator of MHC class II antigen expression on macrophages which extended to ovine MDMs in this study. As T cell sub-populations generally recognise antigen in the context of MHC class I and II molecules (Townsend and Bodmer 1989), this regulation has important implications for initiation and development of the immune response.

IFN-γ increased the expression of MHC class II on MVV-infected MDMs *in vitro*. In contrast, GM-CSF had no enhanced effects on the expression of MHC class II on infected macrophages. AMs of MVV-infected sheep showed increased expression of MHC class II (Luján *et al.* 1993). IFN-γ mRNA was up-regulated in lung tissue of MVV-infected sheep with lung lesions (Woodall *et al.* 1997). These results suggest that IFN-γ induced by MVV *in vivo* may account for the increased expression of MHC class II on infected cell *in vivo*. The increased expression of IFN-γ induces the expression of MHC II on macrophages. The continuous expression of these antigens

might then favour recruitment of lymphocytes into the lung and lead to local inflammation. Thus increased level of cytokines *in vivo* may have damaging rather than favourable effects in the infected microenvironmental sites. Treatment with GM-CSF was able to augment the expression of MHC class I and but not class II by the macrophages. MVV-infected MDMs were found to be positive for MHC class I, GM-CSF in combination with MVV did not show a synergistic effect on induction of MHC class I, thus promoting the hypothesis that GM-CSF may account for the increased expression of MHC class I on MVV infected MDMs *in vitro*.

The continuous expression of CD14 was observed on ovine MDMs. After treatment with GM-CSF, there was no significant increase in the expression of CD14 when compared with unstimulated cells. This finding is inconsistent with a previous study in human monocytes which reported that GM-CSF down-regulates the expression of CD14 on monocytes (Kruger *et al.*1996). This result may reflect a difference in response of different stages of maturation of monocyte to GM-CSF, a cytokine with differentiation-inducing properties on monocytes. GM-CSF thus may have a role in controlling the expression of CD14 and thereby modify certain pathophysological conditions and clinical pictures. IFN-γ was found to exert no influence on the expression of CD14 and CD45. This result was in agreement with the previous report on ovine alveolar macrophages which reported that IFN did not influence that expression of CD45 on macrophages (Nash *et al.*1992). Taken together, the results reported here further support the concept that the changes on expression of surface antigens on macrophages during infection are in part directly related to cytokines.

CHAPTER 7

DISCUSSION

MVV infects monocytes/macrophages and causes persistent infection. However, unlike the human and simian immunodeficiency viruses (HIV and SIV). MVV does not cause immune deficiency but instead cause chronic mononuclear inflammation of various tissues (Gendelman *et al.* 1989, Brodie *et al.* 1995). The lesions are thought to be immune-mediated, but the mechanism involved in their development is poorly understood.

Macrophages are the host cells for the replication of MVV in the lung and synovium (Brodie *et al.* 1992, 1995). MVV-infected macrophages are thought to serve as a stable reservoir for the dissemination and persistence of MVV *in vivo*, therefore the replication of MVV in these cells has been considered as a major factor contributing to chronic inflammatory lesions in the site of disease during MVV infection (Brodie *et al* 1992, 1993, 1995, Luján *et al.* 1994).

Although viral DNA was detected in AM of all animals, irrespective of the histological findings in the lung, there were pronounced differences in the levels of viral burden among animals. Viral load in AM of animal with lung lesions was higher than that in cells of animals without lung lesions. The absolute viral load in AM therefore reflects the pathologic manifestation in the lung. These data, together with the previous studies which reported that animals with moderate or severe lymphoid interstitial pneumonia have high percentage of MVV positive AM (Brodie *et al* 1992; DeMartini *et al* 1993; Luján *et al* 1994), implied that AM as important target cells in the pathogenesis of pulmonary lesions in MVV infected sheep. Thus viral load in AM may have predictive value even if the time of seroconversion is unknown. Serial measurement of viral load over time may provide a therapeutic guidance. It is conceivable that quantitative analysis of viral activity *in vivo* is invaluable in order to understand fully the pathogenic steps of lentiviral infection.

The findings of lower level of MVV DNA load in blood monocytes, even from the sheep with severe lung lesions, when compared with that in AM, suggests that AM may be more susceptible targets than monocytes and represent major focus of MVV infection. The results of an increased susceptibility of *in vitro*-differentiated monocytes to infection with MVV *in vitro*, when compared with that of freshly isolated monocytes provides further support. Thus AM may act as cellular reservoirs of MVV by the time the infected animals develops maedi. MVV would be released from infected AM and then be free to infect other cells within the lung. Thus, increased viral load in AM compared with that in monocytes from sheep with pulmonary lesions also might reflect an inherent increased susceptibility to infection. The mechanism by which differentiated monocytes are more susceptible to productive infection with MVV *in vivo* and *in vitro* is not yet clear.

AM from MVV infected sheep without lung lesions harbour low level of viral DNA. It has been reported that virus production per cell measured by PCR remains stable while the number of infected cells increase with disease progression (Bukrinsky *et al* 1991). Thus the low level of viral load may be a reflection of low number of infected AM in MVV infected sheep without lung lesions. This leads to hypothesis that low proportion of MVV-infected AM in the lung (at early stage of disease) causes the chronic active inflammatory changes with lymphocyte infiltration and proliferation in lung (Lairmore *et al.* 1986, 1988b, Watt *et al.* 1994) through local release of cytokines. It is thought that lentivirus infection results in local release various cytokines and other factors which may in turn recruit mononuclear cells into the inflammatory area, thus providing the increased number of susceptible cells for viral replication (Lairmore *et al.*1986, Zink *et al.*1990). The result of this study showing that the viral DNA burden in AM correlated with lung lesions of disease provides support for such mechanisms.

Although viral DNA was detected in AM of all infected animals, viral RNA was only detected in AM of sheep with lung lesions. No viral RNA was detected in freshly isolated AM from infected sheep without lung lesions, but after *in vitro*

stimulation with TPA, MVV replication was observed in these AM. This suggests a loss in restriction of viral gene expression and replication in AM of animals with prominent lymphoproliferative and inflammatory lesions. It may be considered that AM are latently infected by MVV *in vivo*, and at resting conditions are not a site for viral replication, therefore AM may act ac a latent reservoir for the virus. This leads to the hypothesis that the presence of MVV in the AM of infected animals is likely to represent the consequence of the fact that MVV may reside within AM in a latent state or restricted replication as a provirus integrated within host genomic DNA. The replication of MVV in AM *in vivo* may require factors able to activate viral replication. The result of this study reasserts the importance of the AM as a key cell in the pathogenesis of MVV induced pulmonary disease. Viral RNA was undetectable in AM but detected in spleen and lymph nodes. Thus MVV appears to actively replicate in lymphoid tissue but not in lung although activatable virus is present in lung. The difference may be due to the cell type being infected in these tissues or by the presence of local factors such as cytokines which exert a profound effect on MVV replication. In the lymphoid organs throughout the body, target cells together (with immune complex formation by accessory cells) constitute the ideal milieu for active viral replication This finding promotes a consideration that lymphoid tissues are efficient reservoirs of MVV and a site for viral replication, where target cells may be infected by MVV and transmitted to other tissues such as lung of the body. The localised infected target cells may results in local release various cytokines and other factors which may in turn recruit mononuclear cells including infected target cells into the inflammatory area.

No clear explanations can be forwarded for the difference in the viral replicative status in macrophages. Exposure of macrophages to any differentiation stimuli can regulate viral replication and control of viral persistence (O'Brien *et al.* 1994). A variety of reports demonstrate that cellular factors influence permissiveness of macrophages to productive infection (Gendelman *et al.* 1990) and underscore differences between macrophages. For example, in the case of HIV, low level of interferons or other regulatory factors may be induced in response to HIV infection

in monocytes (Gessani *et al.* 1991). These factors may regulate early stages of virus infection in monocytes/macrophages and may underlie the differences in susceptibility or permissiveness of monocytes/macrophages. Since macrophages are terminally differentiated cells (non-dividing), viral nucleic acids must traverse the nuclear membrane before provirus establishment and viral replication can occur (Weinberg *et al.* 1991). In the case of HIV, nucleophilic components related to the pre-integration complex of HIV-1 facilitate nuclear localisation of viral nucleic acids in the absence of mitosis (Heinzinger *et al.* 1994), thus indicating that restriction to virus replication in macrophages is not at the level of host cell mitosis. The *in vitro* system in which HIV-1 infected macrophages are maintained in culture and exposed to different stimuli has demonstrated that HIV-1 re-enters the replicative cycle in exposure of infected cells to a number of cytokines including IL-1β, IL-6, GM-CSF (for review, see Chapter 1). Thus, another pathway modulating transactivation of lentivirus in macrophages may be related, at least in part, to cytokines network produced during host immune response to viral infection. The comprehension of the regulatory network between MVV and cytokine represent a basic question that awaits elucidation.

MVV infection results in the release of one or more cytokines. A significant increase in expression of IL-6, IL-10, GM-CSF and TGF-β mRNA was observed in AM isolated from naturally infected sheep. TNF-α, IL-6, IL-10 and GM-CSF enhancement was detected in blood monocytes. In LNs, IL-2 and IL-10 was elevated. In particular, the findings that the increased expression of GM-CSF mRNA was observed in AM of MVV infected sheep, may explain, in part, to the previous observations that increased expression of GM-CSF in the lung tissue of MVV infected sheep with chronic lymphocytic interstitial pneumonia (Woodall *et al.* 1997) at cellular level. These results suggest that alterations of cytokine expression may contribute to MVV pathogenesis by regulation of the virus replication in macrophages. The finding that the levels of GM-CSF expression in AM differed in relation to the lung lesions, provides support for such mechanism. It is well known that GM-CSF up-regulates antigen-presenting activity of dentritic cells and recruits

these cell to localised inflamed regions of the lung, thus promoting lymphoid follicles development (Steinman 1991), GM-CSF also mediates abrogation of suppression of T lymphocytes activity in the lung by AM, causing T lymphocytes accumulation to occur. Thus it is possible that increased level of GM-CSF in AM may account for the development of lymphoid follicles in the lung of MVV infected animals. However, *in vitro* studies show that MMV infection does not increase GM-CSF mRNA in macrophages, it is unlikely that the increased expression of GM-CSF *in vivo* is, in part, the direct consequence of MVV infection. It should also be noted that overexpression of cytokines might favour local virus spreading (Agostini *et al.* 1993b). MVV is pathologically similar to HIV. In HIV, GM-CSF is effective at driving virus replication in monocytes (Poli *et al.* 1992b, Crowe and Lopez 1997) and that differentiation and activation of monocytes was observed to be associated with the virus infection and replication. These results suggest that GM-CSF may play a role in driving the HIV replication, thus promoting the spread *in vivo* (Crowe and Lopez 1997). Similar mechanism may exit in MVV infection. The suggestion is supported by that fact that the higher level of GM-CSF mRNA was detected in AM in which MVV RNA and high level of viral DNA was detected. In contrast, MVV RNA was undetectable in AM with the lower level of GM-CSF mRNA and viral DNA level. This response of elevated expression of GM-CSF mRNA may in part account for the observations that a higher viral load was observed in the AM of infected animals with lung lesions and responsible for driving actively replication of the virus in AM. This suggestion is further supported by the *in vitro* study from GM-CSF treated MDMs that GM-CSF has stimulatory effects on MVV replication.

The factors responsible for initiating a switch from asymptomatic to symptomatic disease in lentiviral infections are not yet clear. Th1 and Th2 response in mice play a critical role in viral clearance (Braciale and Braciale 1994). Th1 cytokines are IFN-γ, IL-2 which promote cell-mediated immunity and delayed-type hyper-sensitivity (DTH). Th2 cytokines are IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 secreted by Th2 cells which favour allergic reactions and provide the most efficient help for B cells (antibody production) (Mosmann *et al.* 1986; Romagnani 1994). Several reports

have shown that some infectious agents may be controlled by either Th1 or Th2 response (Khatissian *et al.* 1994, Collette Y *et al.* 1996, Lechner *et al.* 1997). Evidence has suggested that switch in Th1 cytokine to Th2 cytokines response may be responsible for disease progression during HIV infection (Clerici and Shearer 1993). Patients with Th1-type responses, characterised by production of IL-2 and IFN-γ remain as asymptomatic whereas those that switch from Th1 to Th2, as indicated by decreased secretion of IL-12 and increased production of IL-4, develops symptomatic disease (Clerici and Shearer 1993, Chehimi *et al.* 1994). In cats a switch from a Th1 to Th2 response has also been suggested as playing a role in FIV infection based the observation that asymptomatic cats produced high levels of IL-2 and low levels of IL-6. In contrast those in the advanced stages of infection produced low levels of IL-2 and high levels of IL-6 (Lawrence *et al.* 1995). Looking at constitutive cytokine mRNA expression in AM, blood monocytes and LNs of MVV infected animals, there was no evidence to show a demarcation between Th1 and Th2 cytokine profiles. On the basis of this finding, it is hypothesed that both type of cytokine response could occur concurrently during MVV. Thus, studies monitoring changes in levels of Th1 and Th2 cytokines during MVV infection are required before a definite role of a shift from Th1 to Th2 in the immunopathology of MVV infection can be determined. Interestingly, increased levels of IL-6 mRNA were observed in AM, blood monocytes and lymph node, but expression of IL-10 gene was not detected in lymph nodes. This difference may reflect the level of cytokine mRNA expression at the level of single cell type (such as AM) and may not reflect cytokine synthesis in tissue. Whether or not a Th1/Th2 switch occurs, cytokines are certainly critical in modulating the type and the effectiveness of the immune response against lentiviral infection.

The results from this study suggest that the persistent expression of cytokine may also occur because of direct up-regulation by virus or viral glycoproteins. However, the results of this study indicate that the mechanism for the dysregulation of cytokines found in animals with lentiviral infection is more complex. The suggestion is supported by the facts that no expression of IL-6 and IL-10 was observed in

MDMs in response to *in vitro* MVV infection. Apart from MVV, lack of cytokine expression in macrophage in response to *in vitro* infection was also observed in two other types of lentiviruses. A lack of TNF-α expression in response to *in vitro* infection with both HIV (Molina *et al.* 1990) and CAEV (Lechner *et al.* 1996) These findings suggest that this failure may be common feature in lentivirus infection. The mechanism for this behaviour is not yet clear. The results shown in this study suggest two possible mechanisms: (i) Failure to induce IL-10 expression by MVV may be linked to cell differentiation. The results of this study show that IL-10 mRNA was detected in freshly isolated monocytes but not in cultured macrophages in response to MVV infection, provides support for this theory. (ii) A cytokine may have a role in overproduction of another. In this study, GM-CSF was found to stimulate IL-6 expression in sheep MDMs

With regard to MVV infection, the level of GM-CSF expression has been shown to correlate with the severity of lung lesions. GM-CSF was elevated in MVV-infected sheep. Thus it is important to determine the cytokine directly influences the expression of MVV in infected macrophages. Examination of the effects of GM-CSF on MVV replication showed that it was effective at promoting MVV replication in infected MDMs. The enhancing effect of GM-CSF on MVV replication in MDMs is in agreement with reports concerning GM-CSF-stimulated regulation of HIV replication (Folks *et al.* 1987, Koyanagi *et al.* 1988, Perno *et al.* 1989). Collectively these studies are at variance with others that showed the inhibitory effects of GM-CSF on lentivirus expression. Matsuda *et al.* (1995) reported an inhibitory effect of GM-CSF on the replication of HIV in human blood MDMs. Moreover, the studies of Hammer *et al.* (1986) indicated that GM-CSF can suppress viral replication in a promonocytic cell line chronically infected with HIV. This leads to the hypothesis that the regulatory interplay between GM-CSF and lentivirus is complex and may depend on a number of factors including the cytokine milieu and the stage of monocyte differentiation. Nevertheless, the results in this study support the concept that GM-CSF could be an important mediator for promoting MVV replication, thus promoting virus spread.

GM-CSF was observed to increase the total amount of MVV produced in infected macrophages and in this, MVV resembles HIV (Agostini *et al.* 1992). In SIV infection, Walsh *et al.* (1992) have demonstrated that GM-SCF promoted SIV gag protein production. These data suggest that the enhancing effects of GM-CSF on lentivirus replication in macrophages may occur through the stimulation of the production of host-cell transcriptional factors. These might bind to the virus LTR, resulting in transactivation and thus cause an increase in virus transcription. Further experiments were required to clarify this point. Of note is the fact that the level of GM-CSF mRNA was enhanced in MVV-infected macrophages (Chapter 4) and MVV-infected lung (Woodall *et al.* 1997) suggesting that GM-CSF could potentially play a role in enhancing MVV replication in macrophages *in vivo*.

In exploring the effects of TGF-β on MVV replication in macrophages, it was observed that TGF-β inhibited MVV replication of acutely infected MDMs. Whether the inhibitory effects of TGF-β on the replication of lentivirus is direct or indirect mechanism action is still unclear. Many steps have been defined in MVV infection and replication, such as binding to the cell surface, internalisation of the viral core into the cytoplasm, uncoating of viral RNA, reverse transcription, integration of viral DNA to cellular DNA, viral DNA transcription and translation, viral components assembly and virus budding. Inhibition of MVV binding to target ccells by TGF-β seemed unlikely in view of the lack of effects on expression of MHC class II mRNA on macrophages. Further study is required to investigate the specific mechanisms by which TGF-β might inhibit NVV replication after virus binding. Another possible explanation worth investigating could be that TGF-β may exerts its negative effects on the expression of critical cellular proteins involved in the replication of MVV, such as enzymes of the cell signalling pathway that impact on nuclear translocation or other cellular factors which might down-regulate other surface molecules needed for virus binding or inhibit cellular components involved in virus fusion, uncoating or transcription.

The inhibitory effects of TGF-β on MVV replication, together with the observation that the level of TGF-β mRNA was enhanced in MVV-infected macrophages, implies that TGF-β may provide a possible mechanisms for suppression of MVV replication within macrophages and inhibition of the spread of infection. Finally, since others cytokines capable of inducing increased expression of MVV, such as GM-CSF have been found be increased in AM (Chapter 4) or lung tissue (Woodall *et al.* 1997), a delicate balance between stimulatory and inhibitory cytokines is likely to determine the net rate of MVV replication and expression and subsequently in the clinical progression of MVV infection.

Aside from the name, IFN-α, and IFN-γ have little in common. IFN-α is produced by macrophages and B cells and IFN-γ is produced by T cells. There is no-cross reactivity at receptor level. Despite there clear differences, both IFN-α and -γ induce potent antiviral activity against a wide range of retrovirus. However, the role of IFN-α, β, and -γ in the pathogenesis of MVV infection is largely unknown. Previous studies showed the presence of an IFN with biochemical characteristics of both IFN-α and IFN-γ from lung leukocytes from experimentally MVV-infected lambs. As in the *in vitro* study of with 'leukocyte-derived IFN in CAEV-infected monocytes (Zink and Narayan 1989), bovine recombinant IFN-α has a substantially inhibitory effect on MVV replication in macrophages (Ellis *et al.* 1994). In this study, it was not possible to demonstrate any apparent enhanced or inhibitory effects of IFN-γ on MVV replication in MDMs. The reasons for this are not unknown, but may relate to the activation of the infected macrophage at the time of exposure to IFN and the differentiation stage of macrophages on the time of virus infection. *In vitro* culture of blood monocytes results in a time-dependent differentiation into macrophages characterised by an increased expression of certain surface markers (such as CD11/CD18 and CD14 antigens) (Lee *et al.* 1997, Gessani *et al.* 1993). This differentiation process can result in marked change in cell behaviour such as an increased susceptibility to MVV itself (Narayan *et al.* 1982), which may significantly affect virus replication. The above data suggest that a direct effect of endogenous INF-γ on MVV replication or Ag production is unlikely. This may indirectly reflect

the failure of activation of 2',5'A synthease or could be due to the absence in MVV infected cells of other IFN inducible antiviral factors.

In *in vitro* culture systems cytokines have been identified to have profound affect on MVV replication (Chapter 5), however, it is still not unclear whether cytokines have similar regulatory roles *in vivo*. In support of this hypothesis are multiple observations of increased expression of GM-CSF, IL-10, IL-6 and IL-8, and other cytokines potentially capabling of regulating MVV (Woodall *et al.* 1997; Legastelois *et al.* 1997; Chapter 4 and Chapter 5) and HIV (Pantaleo *et al.* 1993, Graziosi *et al.* 1994b, Poli *et al.* 1994).

In conclusion the level of MVV DNA and its replication status in AM from MVV infected sheep and the relationship between virus burden/replication and histological lesions in the lung were assessed. The sheep without histopathological evidence of lesions in the lung, the load of MVV DNA in AM was low and viral RNA was undetectable. Viral RNA became detectable following *in vitro* stimulation of AM with phorbol esters. In contrast, sheep with histopathological lesions in the lung had a significant increase of MVV DNA in AM and viral RNA was detected. These results suggest that the infected AM have a key role in its pathogenesis of lung.

A dysregulation of cytokines was observed in macrophages, blood monocyte and lymphoid tissue of naturally infected sheep. MVV infection changes the pattern of expression of cytokines in macrophages. *In vitro* studies show the enhancing effect of GM-CSF and inhibitory effects of TGF-β on MVV replication in MDM but no effects of IFN-γ, suggesting that cytokines could be an important mediator of regulating the lentiviral replication, Because of the complex network of cytokine interactions, it may be difficult to draw clear-cut conclusion on the role of specific cytokines in MVV in macrophages. Despite these concerns, there is no doubt that cytokines have remarkable potential for the regulation of MVV replication *in vivo* and *in vitro*. A delicate balance between stimulatory and inhibitory cytokines is

likely determining the net rate of MVV replication and expression and subsequently in the clinical progression of MVV infection.

APPENDIX

Appendix I: **Bacterial culture and transformation of *E.coli***

LB -Ampcillin-Methicillin Agar (per litre)

NaCl 10g

Tryptone 10g

Yeast extract 5g

Agar 20g

Deionized H_2O up to 1 litre

Autoclave

Cool to 55°C

Add 20mg of filter-sterilised ampcillin and 80mg of filter-sterilised methicillin. Pour into petri dishes (25ml/100-mm plate)

LB/Amp/Meth/X-Gal plates

LB/Amp/Meth plates were made as above. 50 µl of 20mg/ml X-Gal (made up in dimethyl formamide) was spread directly onto LB/Amp/Meth/ plates with a glass spreader 3 hours before use.

Transformation of competent cells

Supercompetent cells were thawed on ice and transferred into prechilled 15 ml Falcon tube. After adding DNA, the tube was placed in ice for 30 minutes. Cells were then heat-pulsed by putting the tube in a 42°C water bath for 45 seconds and then cooled on ice for 2 minutes. 1 ml of pre-warmed LB/Amp/Meth was added into each tube and incubated at 37°C for 60 minutes. 100ul of the cell culture was spread on LB/Amp/Meth/X-Gal plates which were then incubated overnight at 37°C.

Appendix II: **Preparation of DNA**

Agarose gel electrophoresis

TAE buffer:

50 x TAE

Tirs base	242g
Gacial acetic acid	57.1 ml
0.5M EDTA (pH 8.0)	100 ml

Made up to 1000 ml with deionised water.

Methods

Agarose was dissolved by boiling in 1 x TAE and then allowed to cool a little. Ethidium Bromide was added to a concentration of 1 μg/ml. The gel was poured and left to set. 1 x TAE buffer was made up and added to the tank to just cover the gel. 0.2 volumes of loading buffer were mixed with each sample. Samples were loaded and the gel was run. Nucleic acid in the gels was visualised on an UV tansilluminator.

Alkaline Transfer of DNA

After electrophoresis to separate the DNA fragments, DNA in the gel was denatured by soaking the gel in 0.4N NaOH for 30 minutes. Unnecessary parts of the gel was removed and placed on the top of two pieces of 3MM papers acting as a wick to draw up 0.4N NaOH from the trough below. A piece of membrane cut to exactly the same size as the gel and equilibrated in 0.4N NaOH for 10-15 minutes was placed on the gel. 10 pieces of 3MM papers were placed on the top of the membrane, followed by a stack of dry paper towels. A glass plate and a weight were placed on the top. The assembly was left overnight in a 0.4N NaOH transfer solution. The membrane was washed in an excess of 2 X SSC for 1-2 minutes and air dried.

Hybridization buffer for ^{32}P-labelled probe

dH_2O	84.6 ml
Dextran Sulphate, Na salt (MW 500,000)	10 g

10% SDS 10 ml

Heat at 65°C for 30 minutes, add 0.58 g of NaCl and then heat an additional 15 minutes. This buffer is stored at -20°C until use.

Recovery of DNA from agarose

DNA was recovered from low-melting agarose by binding to then elution from silica. Geneclean reagents were used according to the manufacture's manual. Briefly, DNA sample was run on 1.5% (m/v) low melting agarose gel, and then a slice of gel containing the targeted DNA band was excised and weighed to estimate its volume. 2 volumes of NaI stock solution were added and the gel was melted by heating to 65°C. Glassmilk suspension was added (5μl for 5μg DNA) and placed on the ice for 5 minutes with mixing gently every 1-2 minutes, and then pelleted by centrifuging for 5 seconds. The supernatant was removed and the pellet was washed three times with "NEW WASH". After final washing as much supernatants possible was removed. The DNA was eluted from grassmilk by resuspending the pellet in 10 μl of disionized water and incubating at 50°C for three minutes, followed by centrifuging for one minute(the elution was repeated once more). The eluted DNA concentration was estimated by running a sample on a 2% agarose gel alongside markers.

Minipreps for the isolation of plasmid DNA

Plasmid mini-preps were prepared by using Qiagen Plasmid Spin kits (Qiagen) according to manufacture's instruction. Briefly, Tansformants grown on selective agarose plates were inoculated into LB/Amp./Meth. and grown up for 14 to 16 hours at 37°C with shaking. 1 ml of cell culture was pelleted by 60s microcentrifugation and resuspended in 250 μl of Buffer P1. After resuspension, cell clumps should not be visible. 250 μl of Buffer P2 was added and gently mixed by inverting the tube (dot not vortex), and then 350 μl of Buffer N3 was added and again, inverted immediately but gently. After 10 min micro-centrifugation, the supernatants was transferred to QIAprp column by pipetting. The column was centrifuged for 60s and washed by adding 500 μl of Buffer PB and centrifuging 60s. The column was further washed by adding 750 μl of Buffer PE and centrifuging 60s. After discarding the

flow-throughs and further 1 min micro-centrifugation, the column was placed in clean Eppendorf and 50 µl of H_2O to the centre of the column, let stand for 1 minute and centrifuge for 1 minute. Samples were run on a 1 % agarose gel alongside 1 Kb DNA ladder and the appropriate control plasmid. Regents were prepared following the manufacturer's protocol.

Appendix III: **Sodium-Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis of Proteins**

Proteins were separated by discontinuous polyacrylamide gel electrophoresis with a Tris glycine buffer containing SDS (0.1% SDS, 50mM Tris, 384 mM glycine) (Laemmli, 1970). The resolving gels consisted of either 10 % acrylamide slab gel or 5%-20% linear gradient acrylamide gel (30% acrylamide +1.08% bis acrylamide) in 375mM Tris, pH 8.7 and 0.1% SDS. Stacking gels were 3% acrylamide, 0.15% bisacrylamide in 125mM Tris, pH 6.8 and 0.1% SDS. Samples, mixed with an equal volume of sample buffer, were boiled for 3min. 20ul of the mixture was loaded onto a well (250 µl per large well). Gels were run at 180v for 1hr.and then stained by Coomassie Blue staining for 15 min at room temperature. Coomassie Brilliant Blue G-250 was dissolved in 20% methanol, 5% acetic acid and filtered through Whatman No.1 filter paper. Gels were disdained in several changes of 20% methanol and 5% acetic acid.

Appendix IV: **Detection of Antibodies Specific for MVV**

Preparation of Total MVV Antigens for Western blotting

MVV antigens were prepared from fibroblast culture supernatant by centrifugation. Briefly, the culture supernatant containing virus was harvested and clarified by centrifugation (8500rpm, 4°C, 30min) to remove cell debris and then pelleted by centrifugation (8500rpm, 4°C, overnight). The pellet was resuspended in a minimal volume of PBS and stored in aliquots at -70°Cfor use in immunological experiments. The antigen preparation was assessed by SDS-PAGE.

Western Blotting

The separated proteins on the gel were transferred to pre-wetted nitrocellulose membranes (Hybond C, Amersham) at 120mA/30V for 2 hrs using a semi-dry electroblotter (Ancos, Denmark). The buffer was 48 mM Tris, 39mM glycine and 20% methanol. After transfer, the nitrocellulose membranes were blocked using 5% marvel, 0.05% Tween 20 in PBS at 4°C overnight and incubated with primary antibodies, diluted in PBS/1% Marvel/0.05%Tween 20, at room temperature for 1houre or at 4°C overnight. Blots were washed in PBS /1% Marvel /0.05% Tween 20 three times and then incubated 1 hour with alkaline phosphatase conjugated secondary antibody of appropriate specificity, washed as before and further washed twice in 0.1 M Tris-HCl (pH9.5), Blots were developed with the substrate solution (0.2mg/ml nitro-blue tetrazolium and 0.1mg/ml 5-bromo-4 chloro-3-indolyl phoshpate in 0.1M Tris HCl, pH 9.5 and 0.02M $MgCl_2$ for 10 min or more, as appropriate. The reaction was stopped by washing in water and then dried.

Agar Gel Immunodiffusion Test

AGID test was conducted by a modification of the method of Cutlip et al (1979). Briefly, a microhexagonal well pattern was used with alternating large (40µl) and small peripheral wells (25µl) and a small central well (25µl). Sera from sheep were tested by filling the centre well with antigen (CVL, England) and the large peripheral wells with test sera and the small wells with positive serum (CVL, England). The

plates were incubated overnight at room temperature in a moist chamber, and results were recorded at 24 and 48 hrs.

Appendix V: **Liquid Phrase-blocking ELISA**

96-well plates (Immuno) were coated with 20μg/ml recombinant MVVp25, diluted with 0.05M bicarbonate buffer (pH9.5), overnight at 4°C. The plates were washed at least three times with PBS/0.05% Tween-20 and blocked with 5 % BSA/PBS at room temperature for 1 hour. Samples were incubated with equal volume of pre-titrated monoclonal antibody to p25 overnight at 4°C and then added to each well, incubated at room temperature for 2 hours. Plates were washed at least three times with PBS/0.05% Tween-20. Alkaline phosphatase-conjugated antibody was diluted in 1%BSA/ 0.05%Tween-20 /PBS according to the manufacturer's protocol. 50 μl was added to each well and incubated at room temperature for 2 hours. Plates were washed at least three times with PBS/0.05% Tween-20. 75μl of AP substrates (according to the manufacturer's instruction, made up fresh each time) was added to each well and allowed to develop in the dark at room temperature for 30- 45 minutes. 25 μl of 0.2M NaOH was added to stop the reaction. The optical density was measured at 405nm.

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